

REPLICATION OF HUMAN ADENOVIRUS DNA

by

Bruce W. Stillman

Bruce W. Stillman

Department of Microbiology

John Curtin School of

Medical Research

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## STATEMENT

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Bruce Stillman

Department of Microbiology  
John Curtin School of  
Medical Research.

During the course of this work, I was a recipient of a Commonwealth Postgraduate Research Award.



## A C K N O W L E D G E M E N T S

I wish to sincerely thank Alan Bellett and Ban Younghusband for their support, advice and encouragement during the last three years. It has been a rewarding and valuable experience and I am grateful for the opportunity they provided that enabled me to work on eukaryote molecular biology. I also wish to thank members of the Microbiology Department and other members of the University for their help and friendship.

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During the course of this work, I was a recipient of a Commonwealth Postgraduate Research Award.

## A B S T R A C T

Adenovirus DNA exists in the virion as a linear, double-stranded DNA molecule and also replicates in infected cells in a linear form by a strand displacement mechanism, with origins and termini at each end of the DNA molecule. The mechanism of priming replication at the 5' ends of linear DNA chromosomes remains unclear, as the known DNA polymerases, which act in the 5' to 3' direction, require a free 3'-hydroxyl as a primer for chain elongation. One model for providing a primer for initiation of adenovirus DNA replication involves formation of a hairpin loop at the 3' end of parental DNA, allowing the 3'-hydroxyl to prime DNA replication. A subsequent strand switch and inversion of the terminus by endonuclease nicking and repair synthesis could complete replication of the parental terminal sequence. Three lines of evidence are presented which are inconsistent with this 'hairpin-priming' model for initiation of viral DNA replication.

Adenovirus DNA contains a protein covalently attached to the 5' end of each strand and it has been proposed that this protein is involved in priming of DNA synthesis at the replication origin. In order to determine whether a protein was attached to replicating DNA, an assay system for adenovirus DNA-protein complex on benzoylated, naphthoylated DEAE-cellulose (BND-cellulose) was developed. DNA-protein complex remains strongly bound to BND-cellulose under conditions which elute de-proteinized DNA. The complex can be recovered intact by elution with urea and SDS, or the DNA moiety can be recovered after protease digestion on the column. This assay system has an advantage in that it allows separation of intracellular DNA molecules that were completed during a short pulse of label from those

that were still replicating at the end of the pulse. Using this assay system, it has been demonstrated that a protein is attached, probably covalently, to single-stranded DNA from the terminal restriction fragments of replicating and completed, pulse-labeled viral DNA. Results are also presented which suggest that the protein is made prior to, or at the onset of viral DNA replication. These results are consistent with a proposed function for this protein in initiation of adenovirus DNA replication.

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## PUBLICATIONS

Most of the work reported in this thesis has been published or is about to be published. These papers are:

Stillman, B.W., Bellett, A.J.D. and Robinson, A.J. 1977.  
Replication of linear adenovirus DNA is not hairpin-primed. *Nature*, 269, 723-725.

Stillman, B.W. and Bellett, A.J.D. 1978. Replication of DNA in adenovirus-infected cells. *Cold Spring Harbor Symp. Quant. Biol.*, 43 (in press).

Stillman, B.W. and Bellett, A.J.D. 1979. An adenovirus protein associated with the ends of replicating DNA molecules. *Virology* (in press).

## A B B R E V I A T I O N S   U S E D

Ad	- adenovirus
BND-cellulose	- benzoylated naphthoylated DEAE-cellulose
BUDR	- 5-bromodeoxyuridine
EDTA	- ethylenediamine tetra-acetic acid
GuHCl	- guanidinium hydrochloride
hr	- hour
i.u.	- infectious unit
PBS	- phosphate buffered saline
p.f.u.	- plaque forming unit
PPi	- pyrophosphate
SDS	- sodium dodecyl sulphate
SV40	- simian virus 40
STE	- 0.1M NaCl, 0.001M EDTA, 0.05M Tris, pH 7.2
TE-buffer	- 0.01M Tris pH 8, and 0.01M EDTA
dUMP	- deoxyuridine monophosphate
dUTP	- deoxyuridine triphosphate

Some of the early work on DNA replication in viruses was done by Meselson and Stahl in 1958. They used a density gradient centrifugation technique to show that DNA replication in *Escherichia coli* is semi-conservative. This means that each new DNA molecule consists of one parental strand and one newly synthesized strand. This discovery was a major breakthrough in understanding the molecular basis of heredity.

## CHAPTER 1

### INTRODUCTION - A REVIEW

Some general principles of DNA replication, with specific reference to the replication of linear DNA, are reviewed. This includes the role of the DNA polymerase, the primase, and the helicase. The role of the single-strand binding proteins (SSBs) is also discussed. Finally, the proteins involved in viral DNA replication, stimulation of cell DNA replication and virus-induced transformation are discussed.

## 1.1 DNA structure and general replication principles

With the discovery of the double helical structure of DNA (Watson and Crick, 1953) came the realization of how the DNA might replicate by a semi-conservative manner, later described by Meselson and Stahl (1958). Recent advances have resulted in considerable knowledge of the eukaryotic

Scope of the review. Adenoviruses are oncogenic viruses that can induce tumors in rodents and transform several types of cell in vitro. There are relationships between the viral genes responsible for viral DNA replication, stimulation of host cell DNA replication, and for the induction of the transformed state in some cells that survive infection. In order to understand the process of adenovirus-induced cell transformation, it is necessary to understand the mechanism of initiation of viral DNA replication, the stimulation of host DNA synthesis, and the proteins involved in these processes. This introduction to experimental work on adenovirus DNA replication reviews some general principles of DNA replication, with specific reference to new findings relevant to future chapters, and replication of linear DNA chromosomes, including adenovirus DNA. Nucleic acid-protein complexes that have covalent nucleic acid-protein linkages are also reviewed. Finally, the proteins involved in viral DNA replication, stimulation of cell DNA replication and virus-induced transformation are discussed.

### 1.1 DNA structure and general replication mechanism

With the discovery of the double helical structure of DNA (Watson and Crick, 1953) came the realization of how the DNA might replicate in a semi-conservative manner, later described by Meselson and Stahl (1958). Recent advances have resulted in considerable knowledge of the enzymology and mechanisms of DNA replication and have been reviewed in the past few years (Kornberg, 1974; Denhardt, 1977; Alberts and Sternglanz, 1977). The same basic principles of DNA replication apply to both prokaryotic and eukaryotic cells and their viruses; however, the detailed mechanisms in each individual system can be as variable as the biology of the system itself. I wish to discuss in this section some of the more recent data on general DNA structure and replication which will be pertinent to future discussion in the thesis.

The rapid advances over the past few years in DNA sequencing techniques have resulted in determination of the primary sequence (structure) of several viral genomes. The DNAs of bacteriophages  $\phi$ X174, G4 and fd and of the eukaryotic virus, simian virus 40 (SV40), have been completely sequenced, as have many segments of DNA from other viral and cell chromosomes. This has contributed to major advances in molecular biology (reviewed by Air, 1978). Eukaryotic DNA, including that of animal DNA viruses, is further structured into DNA protein complexes in chromatin. The eukaryotic cell chromosome consists of nucleosome subunits made up of an octamer of histone proteins protecting approximately 140 to 240 base pairs of DNA with a nucleosome core of 140 base pairs. Between each nucleosome unit is a length of spacer DNA that is variable in length between cell types, and even within a single cell type. The structures of chromatin and the nucleosome have been reviewed by Felensfeld (1978).



Replication of double-stranded DNA is semi-conservative, with the progeny molecule containing one strand of parental DNA and one strand of newly replicated DNA. The parental DNA strand provides a template for DNA replication by DNA polymerases, which synthesize DNA by addition of nucleotide monomers in the 5' to 3' direction. Three major species of DNA polymerase have been detected in *E. coli* (Kornberg, 1974), with the *dnaE* gene product, polymerase III, being the enzyme most likely required for in vivo DNA replication. The polymerase I polypeptide has an associated 5' to 3' exonuclease activity that can degrade the non-template strand. Similarly, three major species of DNA polymerase have been detected in vertebrate cells, designated  $\alpha$ ,  $\beta$  and  $\gamma$  (Weissbach, 1975; Denhardt, 1977). Other species of DNA polymerases found in vertebrate cells include mitochondrial DNA polymerase, virus-induced polymerases (Weissbach, 1975) and the unique enzyme class, the RNA-dependent DNA polymerases of the RNA tumor viruses (Temin and Baltimore, 1972). An increase in the levels of  $\alpha$ -polymerase has been correlated with the onset of cell DNA replication (S phase) during the cell cycle and it is most likely that this enzyme is involved in cellular DNA replication. The levels of  $\beta$ -polymerase do not alter during the shift from the resting state (G1 phase) to S phase and a slight rise in  $\gamma$ -polymerase is observed during early S phase.

Due to the antiparallel polarity of the strands in double-stranded DNA, it is clear that within a replication fork in which both parental strands act as templates, one strand must replicate in the 5' to 3' direction overall, while the other replicates in the 3' to 5' direction. To explain replication of a strand in the overall 3' to 5' direction by polymerases that can synthesize only in the 5' to 3' direction, Okazaki and co-workers (Okazaki et al., 1968; Sugino and Okazaki, 1972) proposed the synthesis of short nascent strands in the 5' to 3' direction



(Okazaki fragments) which are subsequently ligated together to form the newly replicated strand. As the known DNA polymerases require a free 3'-hydroxyl group as a primer for elongation of the nucleotide sequence, it was proposed that short oligoribonucleotides are synthesized, used as a primer for the Okazaki DNA fragment, then removed and the gap filled in by repair synthesis using the 3'-hydroxyl from the previous fragment as a primer. In this way, replication of the strand proceeds overall in the 3' to 5' direction. It has been reported (e.g. Machida et al., 1973; Sternglanz et al., 1975; Sternglanz et al., 1976) that elongation of both strands occurs via this discontinuous synthesis mechanism; however, recent examination of this question has shown the situation to be more complex.

Tye et al. (1977) reported that mutants of *E.coli* deficient in the enzyme dUTPase (deoxyuridine triphosphatase), which hydrolyses dUTP to dUMP plus PPi, accumulated short DNA fragments characteristic of Okazaki fragments. These mutants incorporated uracil into DNA which was rapidly removed by an excision-repair process, leading to short DNA fragments. It posed the problem that DNA fragments labeled during a short pulse might not be replication intermediates, as proposed by Okazaki. This effect was reported in an in vitro DNA replication system (Tye et al., 1978a) where reduced levels of dUTPase affected replication of all stages of in vitro  $\phi$ X174 DNA replication, and also in an in vitro replication system for *E.coli* DNA (Olivera, 1978). A similar excision-repair mechanism has been reported for polyoma virus DNA synthesis in isolated cell nuclei (Brynolf et al., 1978) and can contribute to the levels of short DNA fragments observed during replication. However, when DNA synthesis was examined in the absence of uracil excision, using *E.coli* mutants, Tye et al. (1978b) still observed high levels of short DNA fragments, suggesting that these fragments might be involved in DNA replication.

It is now clear that in a wide variety of organisms, discontinuous synthesis of DNA occurs by RNA priming of Okazaki fragments. Okazaki et al. (1975) reported that RNA is covalently linked to the 5' end of short nascent DNA fragments from *E. coli* and that this is not removed in DNA polymerase I mutants (*polA* negative), suggesting that the associated 5' to 3' exonuclease activity removes RNA and the fragments are subsequently joined by DNA ligase. Initiator RNA (Reichard et al., 1974) has also been reported in papovavirus (SV40 and polyoma virus) DNA replication (Magnusson et al., 1973; Reichard et al., 1974; Hunter and Franke, 1974; Anderson et al., 1977), and mammalian cell DNA replication (Tseng and Goulian, 1975, 1977; Wagar and Huberman, 1975).

After taking into account the possibility that short nascent strands were being caused by excision of misincorporated uracil, several groups have demonstrated that Okazaki fragments are synthesized for the elongation of the strand growing in the overall 3' to 5' direction, but not for the strand growing in the 5' to 3' direction. Such asymmetric Okazaki fragment synthesis, called semi-discontinuous replication, has been reported in polyoma virus and SV40 DNA replication (Franke and Vogt, 1975; Flory, 1977; Hunter et al., 1977; Perlman and Huberman, 1977; Kaufmann et al., 1978) and in replication of the *E. coli* chromosome (Olivera, 1978). Previous reports of short nascent DNA strands on the 5' to 3' strand were probably due to excision of uracil during replication.

The short initiator RNA is synthesised by a primer-independent polymerase which is insensitive to rifampicin and other inhibitors that bind to and inactivate RNA polymerases that are required for transcription. The best characterized enzyme is the *dnaG* gene product of *E. coli*, which was named primase (Rowen and Kornberg, 1978a) and similar enzymes have been implicated in synthesis of initiator RNA in polyoma virus

(Eliasson and Reichard, 1978) and bacteriophage T7 DNA replication (Scherzinger et al., 1977). The sequence of enzymic reactions involved in DNA replication in *E.coli* has been obtained by in vitro DNA synthesis using as templates, DNA from the small bacteriophages M13,  $\phi$ X174 and G4 (Kornberg, 1974; Kornberg, 1977; Meyer et al., 1978; Eisenberg et al., 1978; Wickner, 1978). The *dnaG* gene product (primase) can recognize the origin of phage G4 DNA replication and synthesize a short primer. However, in the case of initiation of  $\phi$ X174 replication, additional proteins are required before priming can occur (Wickner and Hurwitz, 1974; McMacken et al., 1977; McMacken and Kornberg, 1978). In this case, five proteins, including the *dnaB* protein, form a replication complex which is recognized by the primase. It was proposed that the *dnaB* protein migrates in a progressive fashion along the template DNA strand and acts as a mobile promoter signal for primase to synthesize primers at a replication fork (McMacken et al., 1978). Thus the primase, together with the *dnaB* gene product, can synthesize primers for Okazaki fragments as well as for initiation of DNA synthesis at the origin of replication in circular DNA bacteriophages. Furthermore, the primase is capable of utilizing both ribo- and deoxyribonucleoside triphosphates and is able to synthesize ribo-, deoxyribo- or mixed ribo-deoxyribonucleotide primers (Wickner, 1977; Rowen and Kornberg, 1978b). The interaction between *dnaB* and *dnaG* proteins at the replication fork is shown in Figure 1.

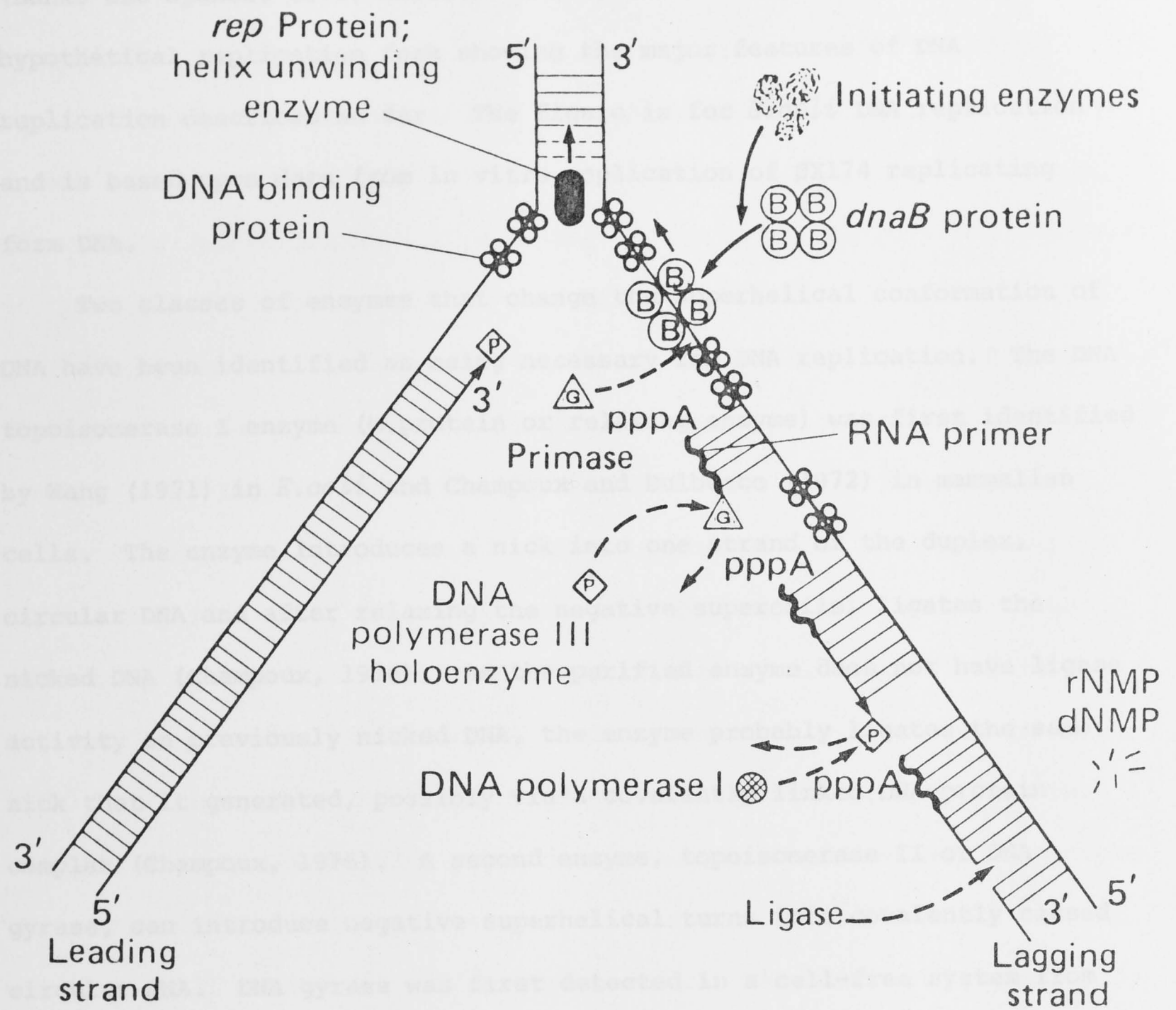
A number of enzymes are involved in separating the two DNA strands at the replication fork to allow DNA synthesis. A protein from *E.coli*, called the *rep* protein, is a DNA unwinding protein that separates strands of DNA just before the replication fork of replicating form  $\phi$ X174 DNA (Eisenberg et al., 1977). Separation of the strands is maintained by a single-stranded DNA-binding protein. Several single-stranded DNA-binding



### Figure 1

A schematic representation of the *E.coli* replication fork showing the proteins required for elongation of DNA chains. The information comes from studies on in vitro replication, mainly using the bacteriophage  $\phi$ X174 or G4 replicating form DNA as a template. The *rep* protein and the DNA binding protein combine to separate the two DNA strands. The *dnaB* protein (B) forms an initiation complex with initiating enzymes, which is then recognized by the primase enzyme (G), the *dnaG* gene product. The primase synthesises a short RNA sequence which is used by DNA polymerase (P) as a primer for chain elongation. On the lagging strand (replication in overall 3' to 5' direction), Okazaki fragments are ligated together by DNA ligase. The replication fork shown here is a semi-discontinuous mechanism for DNA replication.

Figure taken from McMacken et al. (1977).



proteins have been isolated, including the bacteriophage T4 gene 32 protein (Alberts and Fey, 1970; Carroll et al., 1975), the *E.coli* DNA-binding protein (Sigal et al., 1972) and mammalian DNA-binding proteins (Banks and Spanos, 1975; Herrick et al., 1976). Figure 1 shows a hypothetical replication fork showing the major features of DNA replication described so far. The figure is for *E.coli* DNA replication and is based upon data from in vitro replication of  $\phi$ X174 replicating form DNA.

Two classes of enzymes that change the superhelical conformation of DNA have been identified as being necessary for DNA replication. The DNA topoisomerase I enzyme ( $\omega$  protein or relaxing enzyme) was first identified by Wang (1971) in *E.coli* and Champoux and Dulbecco (1972) in mammalian cells. The enzyme introduces a nick into one strand of the duplex, circular DNA and after relaxing the negative supercoils, ligates the nicked DNA (Champoux, 1976). As the purified enzyme does not have ligase activity on previously nicked DNA, the enzyme probably ligates the same nick that it generated, possibly via a covalently linked DNA-protein complex (Champoux, 1976). A second enzyme, topoisomerase II or DNA gyrase, can introduce negative superhelical turns into covalently closed circular DNA. DNA gyrase was first detected in a cell-free system from *E.coli* as being required for integrative recombination of bacteriophage  $\lambda$  DNA using relaxed closed circular DNA as substrate, a process requiring hydrolysis of ATP for the introduction of supertwists (Gellert et al., 1976). Subsequently, DNA gyrase was shown to be a complex of two proteins that were the products of two drug resistance genes in *E.coli* which, when mutated block DNA synthesis in vivo (Sugino et al., 1977; Gellert et al., 1977). These two proteins had four activities. The full complex had DNA gyrase activity (i.e. introduction of supertwists into closed circular



DNA), an associated ATP hydrolysing activity, and under modified conditions, DNA gyrase displayed nicking-closing activity similar to DNA topoisomerase I. Also under modified conditions, DNA gyrase formed a complex with DNA which, upon treatment with the detergent, sodium dodecyl sulphate, caused a double strand break in the DNA with the protein possibly covalently bound to the linear DNA (Sugino et al., 1977; Gellert et al., 1977). The gyrase subunits of both the *E.coli* enzyme (Higgins et al., 1978) and the *Micrococcus luteus* enzyme (Liu and Wang, 1978) have been purified and shown to have these activities in a re-constituted complex.

Two types of protein involved in phage and plasmid DNA replication have interesting functions in initiation of DNA replication and have similar properties to the DNA topoisomerases. The  $\phi$ X174 gene A product, or cistron A protein, introduces a single-stranded nick into superhelical replicating form (RF1) DNA at the origin of DNA replication and becomes covalently attached to the 5' end of the nicked viral strand (Figure 2). Replication proceeds via a rolling circle mechanism (Gilbert and Dressler, 1969), utilizing the free 3'-hydroxyl of the viral DNA strand, when the *rep* protein, the DNA-binding protein and the DNA polymerase III holoenzyme (Kornberg, 1974) are added to the in vitro system. The cistron A protein, which is still bound to the 5' end of the viral DNA strand, migrates with the replication fork to aid in separating the duplex DNA. When replication has gone the full circle, the cistron A protein ligates the newly replicated strand to form a circular viral molecule utilizing the proposed second active site on each cistron A protein (Eisenberg et al., 1977). This series of events has been described by Eisenberg et al. (1977), and is shown in Figure 2. If the polIII holoenzyme is left out of the reaction, the cistron A protein plus the *rep* protein and DNA-binding protein retain the ability to separate strands in an 'abortive'

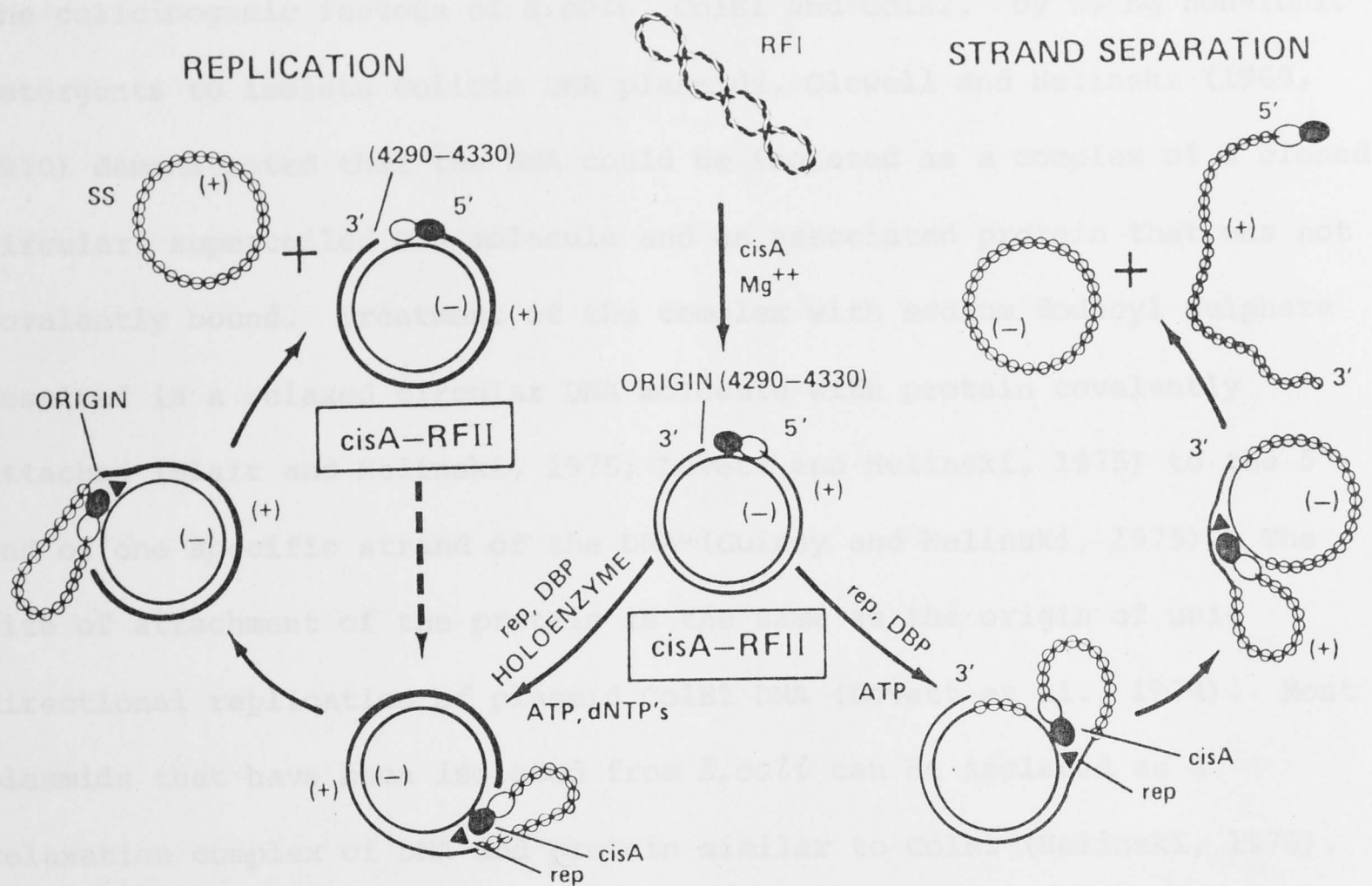
## Figure 2

A schematic representation of the involvement of the  $\phi$ X174 cistron A protein in in vitro DNA replication (left) or in vitro strand separation (right). The cistron A protein starts a round of replication by nicking the double-stranded, supercoiled replicating form 1 (RF1) molecule at the origin of viral strand DNA synthesis (between nucleotides 4290-4330 on the  $\phi$ X174 sequence map). The nicking results in covalent attachment to the 5' end of the viral DNA strand (+).

In the replication reaction (shown on the left), addition of the *rep* protein, DNA-binding protein (DBP), ATP, the four deoxynucleotide triphosphates and the polymerase III holoenzyme allows synthesis of a new viral (+) strand (thick line) and displacement of the parental viral (+) strand which is covered with the DBP (o). The cistron A protein may have two active sites or subunits (indicated by the open and closed circles), the second site acting as a DNA ligase to form a covalently closed, single-stranded viral DNA molecule.

Alternatively, if the holoenzyme is left out of the reaction, the cistron A protein can form an 'abortive' replication fork and unwinds the two strands without net DNA synthesis (shown on the right).

Taken from Eisenberg et al. (1977).





replication fork (Figure 2). The cistron A protein is also required for synthesis of more replicating form DNA as well as single-stranded progeny viral DNA (Eisenberg et al., 1976; Sumida-Yasumoto et al., 1976).

A protein is covalently associated with the relaxed form of DNA from the colicinogenic factors of *E.coli*, ColE1 and ColE2. By using non-ionic detergents to isolate colicin DNA plasmids, Clewell and Helinski (1969, 1970) demonstrated that the DNA could be isolated as a complex of a closed circular, supercoiled DNA molecule and an associated protein that was not covalently bound. Treatment of the complex with sodium dodecyl sulphate resulted in a relaxed circular DNA molecule with protein covalently attached (Blair and Helinski, 1975; Lovett and Helinski, 1975) to the 5' end of one specific strand of the DNA (Guiney and Helinski, 1975). The site of attachment of the protein is the same as the origin of unidirectional replication of plasmid ColE1 DNA (Lovett et al., 1974). Most plasmids that have been isolated from *E.coli* can be isolated as a relaxation complex of DNA and protein similar to ColE1 (Helinski, 1976). The protein covalently bound to the relaxation complex has been implicated in specific initiation of DNA replication of plasmid ColE1 and also in providing the nicking-closing activity necessary for replication of supercoiled plasmids (Helinski et al., 1975; Helinski, 1976).

## 1.2 Initiation at the origin of DNA replication

The strategy of initiation and replication varies between different DNA chromosomes. Some DNA chromosomes that replicate as a covalently closed circular molecule have a single origin and replication proceeds in both directions away from that origin. The original model described by Cairns (1963) proposed that the *E.coli* chromosome replicated as a  $\theta$  structure from a unique origin. This was subsequently shown to be

bi-directional in *E.coli* (Bird et al., 1972) and in *Bacillus subtilis* (Wake, 1972). Schnös and Inman (1970) and Valenzuela (1976) have demonstrated by electron microscopy that bacteriophage  $\lambda$  DNA replicates bi-directionally from a single origin until the replication forks meet at a non-unique terminus. The DNAs of the small papovaviruses, SV40 and polyoma, also replicate bi-directionally from a fixed origin (Bourgau and Bourgau-Ramoisy, 1971; Danna and Nathans, 1972; Fareed et al., 1972; Crawford et al., 1973; Kasamatsu and Vinograd, 1974). The newly replicated DNA appears as a relaxed molecule, attached to the supercoiled non-replicated parental DNA (Roman et al., 1974).

Several circular DNA chromosomes have a single origin, but only replicate DNA in one direction from that point. Examples are the single-stranded DNA phages  $\phi$ X174, M13 and G4 for complementary strand, replicating form and viral strand synthesis (Weisbeek and van Arkel, 1976; Kornberg, 1974; Martin and Godson, 1977). However, there is a difference between  $\phi$ X174 and G4 for the synthesis of the viral complementary strand, which is the first stage of replication, in that G4 has a single unique origin of replication (Bouché et al., 1975; Martin and Godson, 1977), whereas  $\phi$ X174 has a single random origin of replication (Eisenberg and Denhardt, 1974). Plasmid ColE1 DNA also has a unique origin and replication is uni-directional (Helinski, 1976). Another example is the replication of mitochondrial DNA, which starts at a specific origin on one strand of the closed circular molecule and proceeds via a displacement loop (D-loop) in one direction (Kasamatsu and Vinograd, 1974). The displaced strand is later replicated from another origin before the molecules separate.

Finally, several chromosomes have multiple origins and bi-directional replication. Examples are the phages T4 (Miller, 1975) and T5

(Bourguignon et al., 1976) and the eukaryote chromosome. Huberman and Riggs (1968) demonstrated that eukaryotic DNA has multiple replication units (replicons) which replicate bi-directionally and have variable size. The number of replication origins and their times of activation vary, depending upon the cell type, as does the rate of replication at the fork during S phase and with the addition of extrinsic factors such as estrogen (see Denhardt, 1977).

The nature of the origin of DNA replication itself has been examined in a number of chromosomes recently, using DNA sequencing techniques. The origins of the bacteriophages  $\phi$ X174 (Langeveld et al., 1978), fd (Gray et al., 1978; Geider et al., 1977), G4 (Godson, 1978) and  $\lambda$  (Denniston-Thompson et al., 1977) have been sequenced, as have the origin of the *E.coli* plasmid ColE1 (Tomizawa et al., 1977) and the origins of the eukaryotic viruses SV40 (Subramanian et al., 1977), polyoma (Freidmann et al., 1978) and adenovirus (Steenburgh et al., 1978; J.R. Arrand and R.J. Roberts, personal communication). The origin of complementary DNA synthesis in bacteriophage G4 has approximately 50% of the origin identical to the phage  $\lambda$  origin and the origins of G4 and  $\phi$ X174 viral strand synthesis are similar. The origins of replication of the related papovaviruses, polyoma and SV40, have similar regions within the sequence. Within most sequences at the origins of replication, considerable secondary structure can be proposed (Denniston-Thompson et al., 1977; Subramanian et al., 1977), but more comparative sequences will be required before any indication of their biological relevance can be obtained.

The priming of DNA replication at the origin is for the most part similar to priming of Okazaki fragments, with the involvement of either an RNA polymerase or a DNA primase. For the priming of  $\phi$ X174



complementary DNA synthesis, more proteins are required to form a pre-priming initiation complex than are needed for Okazaki fragment priming (Meyer et al., 1978). The SV40 tumor antigen (T antigen) is known to bind specifically to the origin of SV40 DNA replication (Tjian, 1978) and has a role in viral DNA replication (see section 1.10). The study of initiation of DNA replication in eukaryotic systems has been held up by the lack of an in vitro system which will initiate DNA replication. One possible system for studying eukaryote DNA replication, including origin-specific initiation, is in a soluble extract from unfertilized eggs of the frog, *Xenopus laevis*, using as a template, a supercoiled ColEI-type plasmid which contains *Xenopus laevis* DNA sequences (Benbow et al., 1978).

Not all priming of DNA replication occurs by the synthesis of ribo- or deoxyribo-oligonucleotides. In the special case of DNA synthesis by reverse transcriptase in the RNA tumor viruses, synthesis of the complementary DNA strand is primed by a host-coded transfer RNA (tRNA) (Harada et al., 1975). The linear DNAs from the parvoviruses have a terminal palindromic sequence that can form a hairpin loop at the origin of DNA replication. DNA synthesis is primed by the 3'-hydroxyl on the parental (template) strand (see section 1.3), with subsequent cleavage of that strand opposite the origin and repair synthesis to regenerate its 3' end.

### 1.3 Replication of linear DNA chromosomes

The chromosomes from nearly all bacteriophages that contain double-stranded DNA have been isolated as linear molecules (Thomas and McHattie, 1967; Kleinschmidt, 1969). However, an exception is the duplex DNA isolated from the bacteriophage PM2 which exists as a covalently closed circular DNA in the phage particle (Espejo et al., 1969).

Linear duplex DNAs have also been isolated from the virions of many viruses of eukaryotes. Herpes simplex virus type 1 contains a linear duplex DNA (Roizman and Spear, 1973; Grafstrom et al., 1975; Wadsworth et al., 1975; Skare and Summers, 1977), as does vaccinia virus, a typical poxvirus (Easterbrook, 1967; McCrea and Lipman, 1967; Becker and Sarov, 1968; Dales, 1973), whose complementary strands are naturally crosslinked (Berns and Silverman, 1970; Geshelin and Berns, 1974; Holowczak, 1976). The DNA from adenoviruses is also isolated as a linear duplex DNA molecule (Green et al., 1967; van der Eb et al., 1969; Green, 1970; Philipson et al., 1975). Unlike higher eukaryotes, the unicellular organisms *Tetrahymena pyriformis* and *Paramecium* sp. contain linear mitochondrial DNA (Suyama and Miura, 1968). A group of eukaryote viruses, the parvoviruses, contain linear DNA, but in the virion the DNA is single-stranded (Mayor, 1973).

The mechanism of replication at the 5' ends of these linear DNA molecules remains unclear (Watson 1972; Bellett and Younghusband, 1972), as the known DNA polymerases require a free 3'-hydroxyl as a primer for chain elongation. If a short ribonucleotide sequence primed DNA synthesis, as for priming of Okazaki fragment synthesis, its subsequent removal would leave no primer for gap fill synthesis at the 5' end. As was seen in earlier sections, replication of DNA in a covalently closed circular structure avoids this problem. With a bi-directional mechanism for replication on covalently closed circular DNA, termination of replication occurs when both growing forks meet. This problem is also avoided with a unidirectional replication mechanism, where the primer can be excised and DNA synthesized in its place after replication has gone the full circle. In both cases, the origin of DNA replication is replicated by a similar mechanism to that used for other parts of the

molecule. Most linear, bacteriophage DNA molecules therefore have terminal repeats or single-stranded complementary ends which allow formation of circular or concatemeric replication intermediates. These two mechanisms will be discussed in detail using as examples bacteriophage  $\lambda$  and bacteriophage T7. A third mechanism for overcoming the 5' end replication problem, self-priming by hairpin loops, will be discussed in section 1.4 and is relevant to the linear DNA molecules of eukaryotes.

The linear  $\lambda$  DNA molecule rapidly circularizes upon entry into a cell, aided by the existence of cohesive single-stranded ends 12 bases long that allow annealing between left and right ends of the molecule (Davidson and Szybalski, 1971; Yarmolinsky, 1971; Kaiser, 1971). The circular molecule then undergoes a few rounds of replication, replicating bi-directionally as a covalently closed supercoiled molecule with relaxed circular progeny DNA (Schnös and Inman, 1970; Kaiser, 1971; Sogo et al., 1976). This early mode of replication is analogous to replication of other covalently closed circular molecules such as bacterial plasmids, polyoma and SV40 DNA and bacterial chromosomes. The newly replicated duplex circles of  $\lambda$  DNA are not substrates for packaging into virions (Sogo et al., 1976), but are used for further replication of viral DNA by a rolling circle mechanism to produce long linear DNA molecules, or concatemers, which are many times the length of a viral DNA molecule (Carter et al., 1969; Wake et al., 1972; Skalka et al., 1972; Takahashi, 1974; Bastia et al., 1975). The resulting linear concatemers of  $\lambda$  DNA are converted to linear viral length DNA molecules with cohesive ends, which are then packaged into phage particles. This requires the function of genes coding for phage head proteins (Wake et al., 1972).

The bacteriophage P22 also replicates its DNA by a rolling circle mechanism. The DNA of P22, like that of the T even bacteriophages



(e.g. T4) is circularly permuted. The physical ends of the linear molecule can occur anywhere within the genetic map and the DNA is also terminally redundant (Rhoades et al., 1968). Terminal redundancy on a DNA molecule is where a sequence at one end of the linear molecule is repeated in the same orientation at the other end. Cohesive ends have to be exposed by an exonuclease, such as the 3' exonuclease III from *E.coli* for in vitro circularization of the DNA to occur. The linear DNA molecule from phage P22 is able to circularize by a recombination event in the cell, replicate via a rolling circle mechanism and form concatemers (see Suskind and Botstein, 1978, for a review). The DNA is matured and packaged by the 'head-full' mechanism (Streisinger et al., 1967) in which the concatemers are encapsulated into the phage particle and the length of the mature DNA is determined by the amount of DNA that can be packaged. Since the genome is smaller than the amount that can be packaged, the genome has terminal repetitions and is circularly permuted (Suskind and Botstein, 1978).

A second mechanism to overcome the problem of replication at the 5' end of linear DNA molecules is via a concatemeric intermediate as in the replication of bacteriophage T7. The DNA from bacteriophage T7 has a terminal repetition of approximately 260 base pairs, but unlike the T even phages, the DNA is not circularly permuted (Ritchie et al., 1967). Upon entering the cell, T7 DNA starts replicating as a linear chromosome from a preferential initiation site 17% from the left hand end. Replication proceeds in both directions until the replication forks reach each end, producing two unit length daughter molecules (Wolfson et al., 1972; Dressler et al., 1972). Initiation can often occur on an already replicating Y-shaped molecule at the 17% initiation site (Dressler et al., 1972). However, the 5' end of each newly replicated strand in these replicating linear molecules could not be completed due to the lack of a suitable primer (Watson, 1972), thus leaving single-stranded tails at each end of



the molecule. The fact that T7 DNA is terminally repetitious, led Watson (1972) to propose that replication of the 5' end is accomplished by the formation of linear concatemers, that is, by end-to-end interaction of the complementary single-stranded DNA that is produced by the inability to replicate the 5' end of the linear DNA during the first rounds of replication (Figure 3). Large fast-sedimenting concatemers of T7 DNA have been observed (Kelly and Thomas, 1969; Center, 1972; Serwer, 1974; Frölich et al., 1975; Paetkau et al., 1977; Langman and Paetkau, 1978). The replicative intermediates are associated with membranes and protein (Pacumbaba and Center, 1973), but can be isolated as large concatemers of DNA without associated membrane and protein (Serwer, 1974; Paetkau et al., 1977; Langman and Paetkau, 1978). Recently, Langman et al. (1978) have demonstrated by restriction enzyme analysis that the concatemers are linear genomes linked in a head-to-tail arrangement and adjacent genomes within a concatemer share a copy of the terminal repetition and therefore are partially overlapping (Figure 3). Maturation of the DNA into genome length molecules probably occurs by single-strand nicking of concatemers and repair of the 3' end of the DNA (Watson, 1972). An overall scheme for completion of the 5' end of T7 DNA during replication is shown in Figure 3.

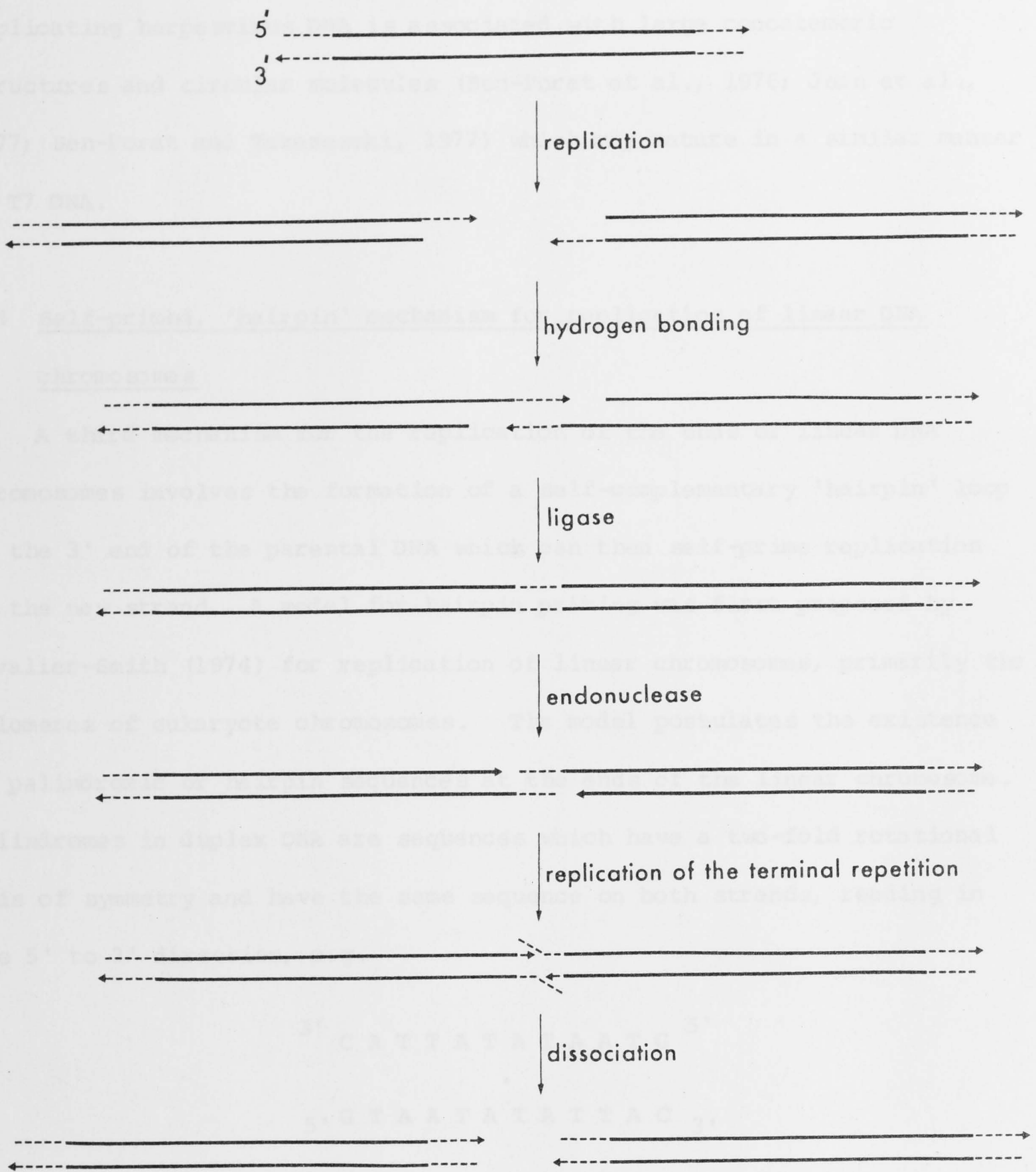
Herpesviruses contain linear duplex DNA with terminally repetitive sequences (Wadsworth et al., 1976) and it has been shown that the initial stage of replication occurs via a linear replicating structure (Shlomai et al., 1976; Hirsch et al., 1977; Jean et al., 1977; Ben-Porat and Tokazewski, 1977) that resembles the early stage of T7 DNA replication. The linear parental and progeny DNA contain single-stranded ends, which allows circularization and formation of concatemers (Jean and Ben-Porat, 1976). The existence of single-stranded ends on parental DNA suggests

Figure 3

A model for the completion of the 5' ends during replication of bacteriophage T7 DNA, proposed by Watson (1972). The terminal repeated sequence is indicated by the dashed lines and the unique sequence by the solid lines. The 3' ends of the DNA are indicated by the solid triangles.

The first rounds of replication produce cohesive single-stranded ends on the linear DNA molecules due to the inability to replicate at the 5' end of the DNA. After inter-molecular hydrogen bonding of the complementary sequences and ligation to form concatemers, the DNA is nicked by an endonuclease opposite these joins. The resulting 3' hydroxyl ends are used as primers for repair synthesis of the terminal repeats and the completed DNA molecules dissociate.

## T7 DNA



that an exonuclease is involved in forming these structures, rather than replication, as in the case of T7 DNA replication. At a later stage, replicating herpesvirus DNA is associated with large concatemeric structures and circular molecules (Ben-Porat et al., 1976; Jean et al., 1977; Ben-Porat and Takazewski, 1977) which may mature in a similar manner to T7 DNA.

#### 1.4 Self-primed, 'hairpin' mechanism for replication of linear DNA chromosomes

A third mechanism for the replication of the ends of linear DNA chromosomes involves the formation of a self-complementary 'hairpin' loop at the 3' end of the parental DNA which can then self-prime replication of the new strand. A model for hairpin priming was first proposed by Cavalier-Smith (1974) for replication of linear chromosomes, primarily the telomeres of eukaryote chromosomes. The model postulates the existence of palindromic or hairpin sequences at the ends of the linear chromosome. Palindromes in duplex DNA are sequences which have a two-fold rotational axis of symmetry and have the same sequence on both strands, reading in the 5' to 3' direction, e.g.



where the axis of symmetry passes through the dot. Each single-strand from the palindrome is capable of forming a hairpin structure by base pairing with itself. Other hairpin sequences are similar to palindromic sequences except that they contain regions which do not base pair, usually in the middle, forming a single-stranded loop. Palindromic sequences are quite common in eukaryotic DNA (Cavalier-Smith, 1976). The model also postulates the existence of specific endonucleases which can recognize one end of the sequence and cleave the correct strand.

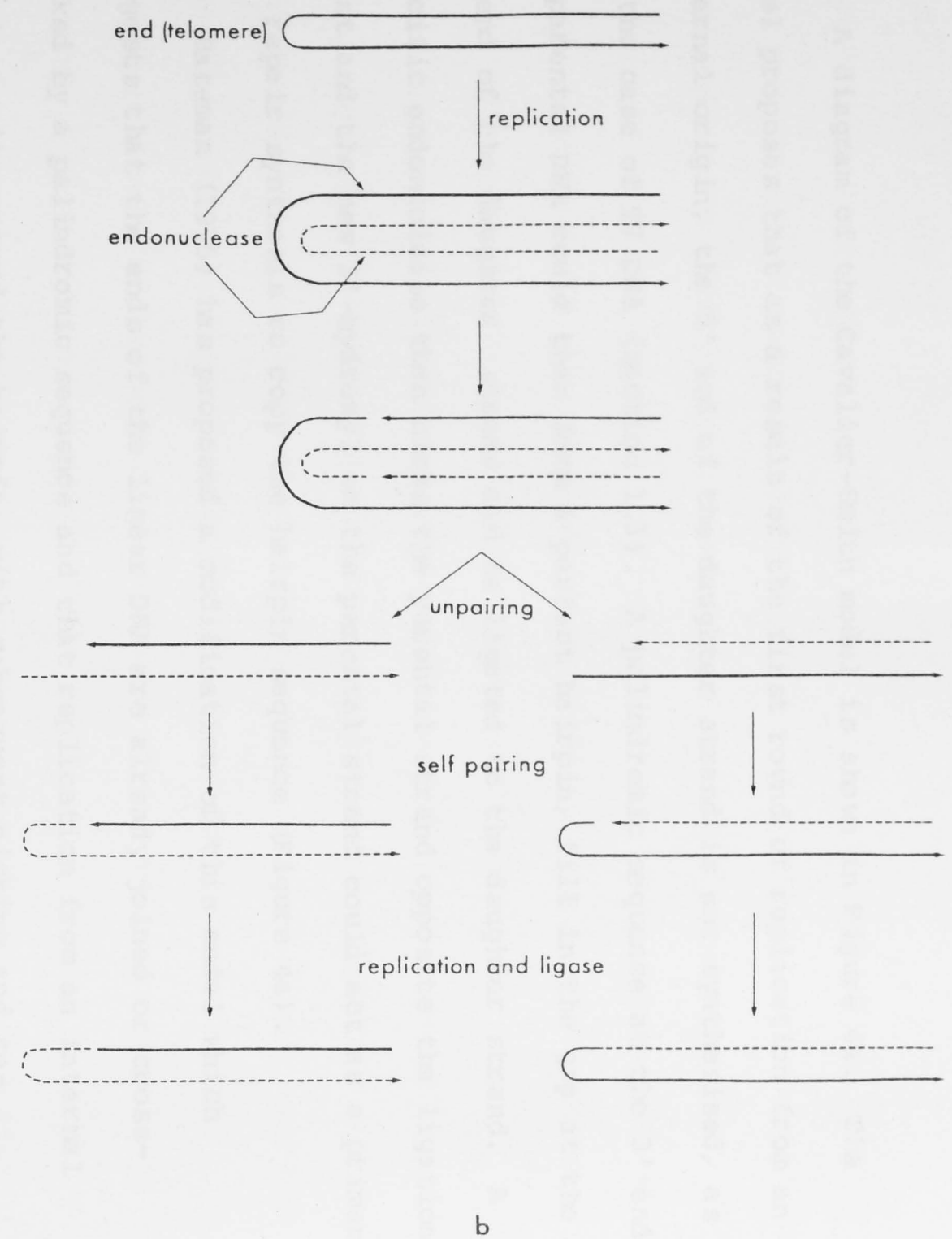
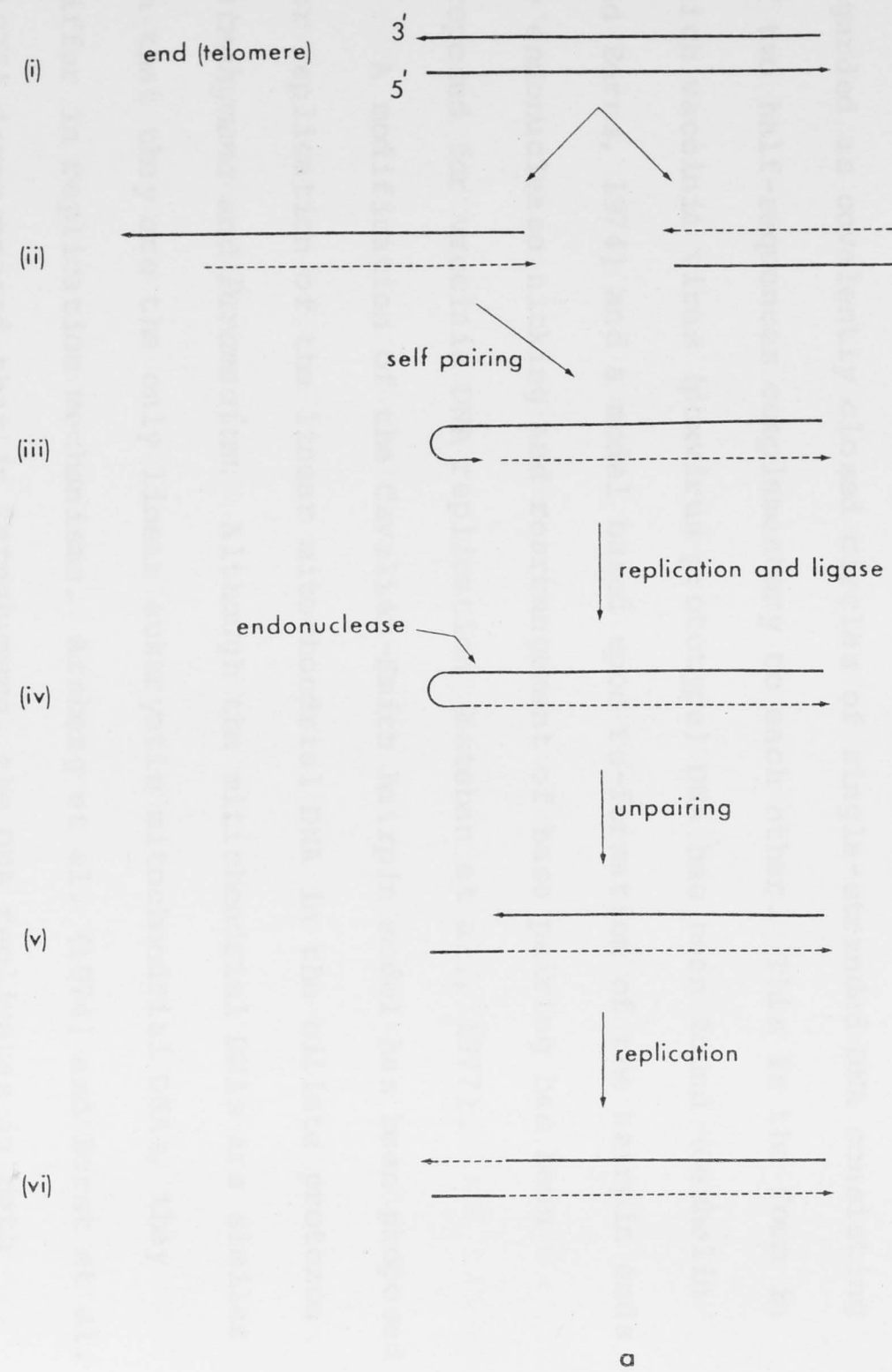


Figure 4

Models for the replication of the ends of linear DNA by self-priming by a hairpin loop. (a) Model proposed by Cavalier-Smith (1974).

- (i) The end of the linear DNA is replicated from an internal origin;
- (ii) resulting in a gap at the 5' end due to the lack of a primer.
- (iii) A self-complementary hairpin loop is formed by the parental DNA, which is ligated (iv) to the daughter strand. (v) An endonuclease nicks the parental strand and (vi) replication of the 3' end can occur to complete the end.

(b) A modified version of the model proposed by Bateman (1975). This model proposes that the ends of the linear DNA are joined in a hairpin crosslink and after replication, a similar strand switch mechanism occurs. Instead of the new 3' end being synthesized, the ends of the daughter DNA molecules are crosslinked by a hairpin.



A diagram of the Cavalier-Smith model is shown in Figure 4a. The model proposes that as a result of the first round of replication from an internal origin, the 5' end of the daughter strand is not synthesised, as in the case of T7 DNA (section 1.3). A palindromic sequence at the 3' end of parental DNA could then form a perfect hairpin, fill in the gap at the 5' end of the daughter strand and be ligated to the daughter strand. A specific endonuclease then nicks the parental strand opposite the ligation point and the new 3'-hydroxyl on the parental strand could act as a primer for repair synthesis to copy the hairpin sequence (Figure 4a).

Bateman (1975) has proposed a modification of this model which suggests that the ends of the linear DNA are already joined or cross-linked by a palindromic sequence and that replication from an internal origin copies around the hairpin, with subsequent nicking and rearrangement to form cross-linked ends again (Figure 4b). This cross-linking would mean the linear double-stranded DNA molecules would also be regarded as covalently closed circles of single-stranded DNA consisting of two half-sequences complementary to each other. This is the form in which vaccinia virus (poxvirus prototype) DNA has been found (Geshelin and Berns, 1974) and a model based upon re-formation of the hairpin ends by endonuclease nicking and rearrangement of base pairing has been proposed for vaccinia DNA replication (Esteban et al., 1977).

A modification of the Cavalier-Smith hairpin model has been proposed for replication of the linear mitochondrial DNA in the ciliate protozoa *Tetrahymena* and *Paramecium*. Although the mitochondrial DNAs are similar in that they are the only linear eukaryotic mitochondrial DNAs, they differ in replication mechanisms. Arnberg et al. (1974) and Borst et al. (1975) demonstrated that in *Tetrahymena*, the DNA replicates in both directions from an internal origin, resulting in two linear progeny molecules which have to complete the 5' ends of the newly synthesized

daughter strands. They proposed a model for replication of the ends of the DNA (Figure 5) which involved formation of a hairpin structure at one end of the linear molecule and then a 'pan-handle' structure by hybridization of an inverted terminal repetition. After conversion of this structure into a covalently closed circle with a single-stranded gap, DNA synthesis and ligation of the original ends could occur, resulting in a covalently closed double-stranded circular molecule. This could then be converted to linear double-stranded molecule (Figure 5) by a double-stranded endonuclease. This model only suggests how one progeny molecule is replicated. It would be necessary to propose that a hairpin exists on both ends of the molecule if net DNA synthesis were to occur.

The linear mitochondrial DNA from *Paramecium aurelia* replicates from an origin at one end of the linear molecule (Goddard and Cummings, 1975, 1977) to produce linear dimers. Goddard and Cummings (1975 and 1977) proposed that initiation occurred near the end and that the two parental strands are cross-linked. Replication could then proceed around the hairpin end, producing linear dimers (Goddard and Cummings, 1977). The linear progeny molecules would be produced by endonuclease action and repair synthesis. However, a linear dimer is unable to replicate the 5' ends of its progeny strand just as the original monomer was, and one of the daughter strands would be incomplete. Thus, the model proposed by Goddard and Cummings (1977) would still require some other mechanism to replicate one end of its DNA, possibly in a similar way to that proposed for the linear mitochondrial DNA from *Tetrahymena*.

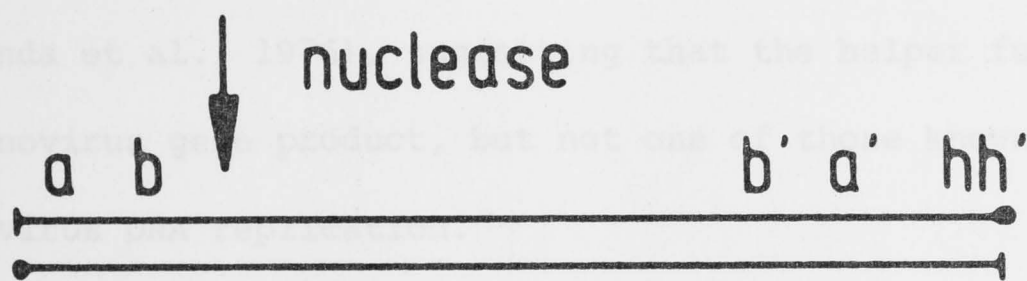
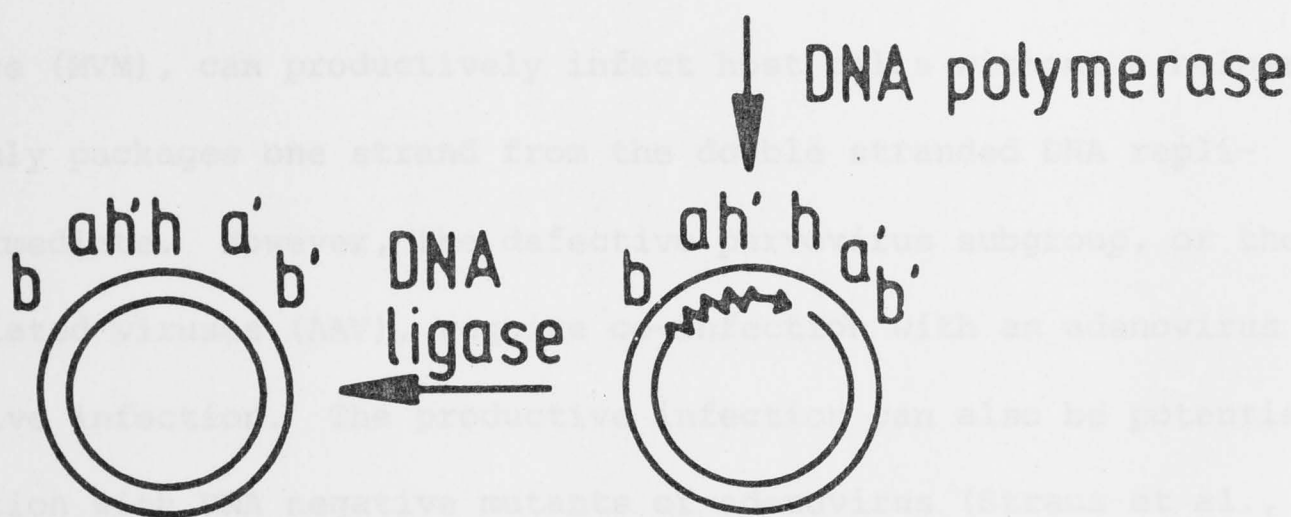
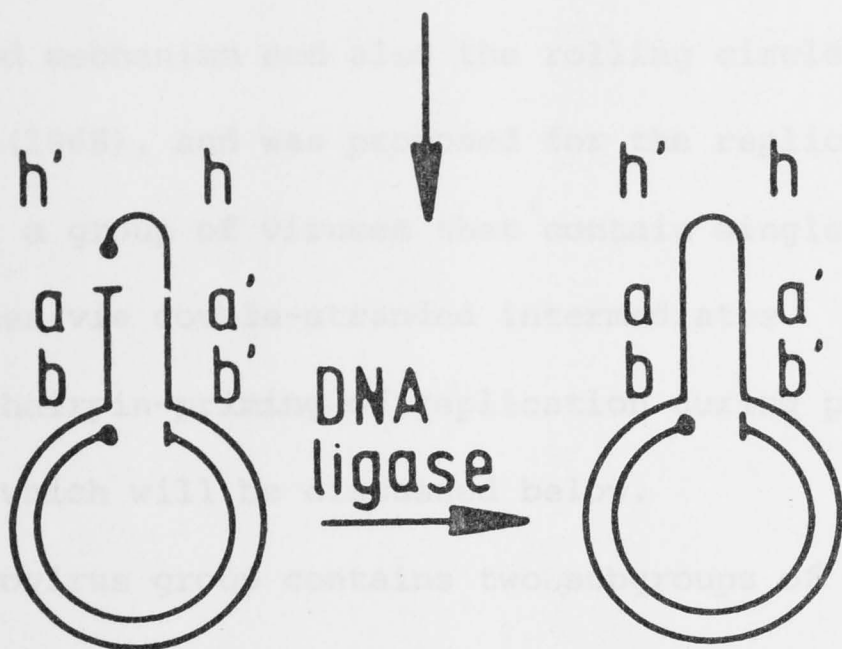
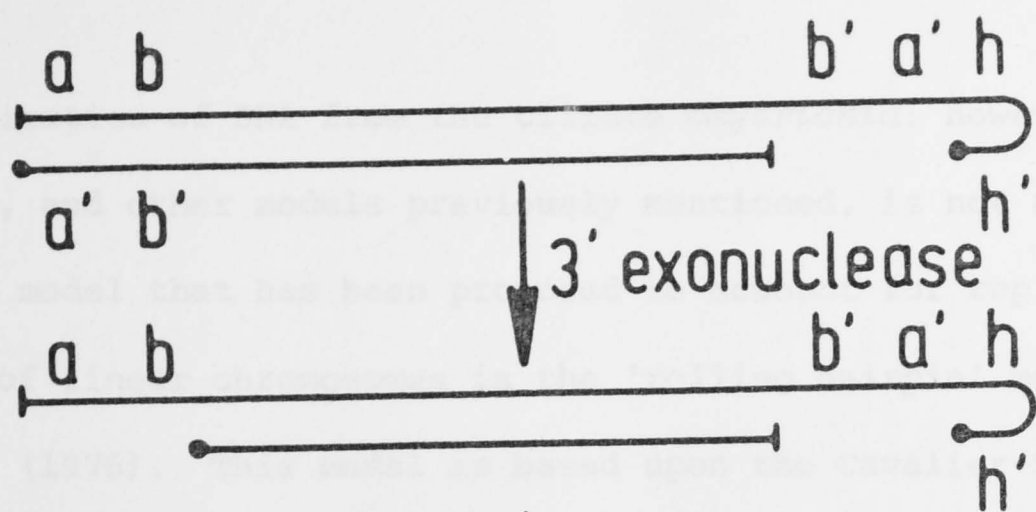
Another modification of the hairpin-primed model has been proposed by Heumann (1976) in which internal repeats of the terminal sequences are involved in strand switching to replicate the ends of linear chromosomes. This could occur in herpesvirus replication and



Figure 5

A model for the replication of linear *Tetrahymena* mitochondrial DNA. The formation of a hairpin structure by the hypothetical h/h' sequence and circularization by the inverted terminal repetition (ab) could form a covalently closed double-strand circle to synthesize the 5' end of the new daughter strand. The 3' ends are indicated by dots.

Taken from Borst et al. (1975).



and replication of DNA from the ciliate *Oxytrichia*; however, evidence for this, and other models previously mentioned, is not available.

One model that has been proposed to account for replication at the 5' ends of linear chromosomes is the 'rolling hairpin' model of Tattersall and Ward (1976). This model is based upon the Cavalier-Smith (1974) hairpin-primed mechanism and also the rolling circle mechanism of Gilbert and Dressler (1968), and was proposed for the replication of the DNA from parvoviruses, a group of viruses that contain single-stranded linear DNA that replicates via double-stranded intermediates. There is considerable evidence for hairpin-priming of replication during parvovirus DNA replication, which will be discussed below.

The parvovirus group contains two subgroups of viruses. The non-defective or autonomously replicating subgroup, which includes the minute virus of mice (MVM), can productively infect host cells without a helper virus and only packages one strand from the double stranded DNA replication intermediate. However, the defective parvovirus subgroup, or the adeno-associated viruses (AAV), require co-infection with an adenovirus for productive infection. The productive infection can also be potentiated by co-infection with DNA negative mutants of adenovirus (Straus et al., 1976b; Handa et al., 1976), suggesting that the helper function is an early adenovirus gene product, but not one of those known to be required for adenovirus DNA replication.

The AAV subgroup can package either strand from the double-stranded replicative intermediate. Thus there are two types of AAV virions which contain either the plus or the minus single-stranded DNA molecules (Berns, 1974). The structure of the single-stranded virion DNA for the non-defective parvoviruses is shown in Figure 6. The structure of AAV DNA is similar, but the DNA contains an inverted terminal repetition. The unique structure of parvovirus DNA (discussed in detail below) led

Figure 6

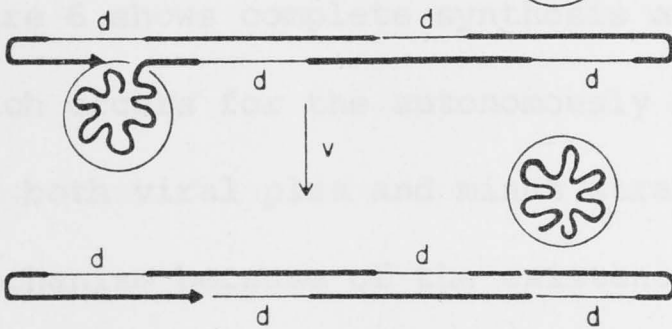
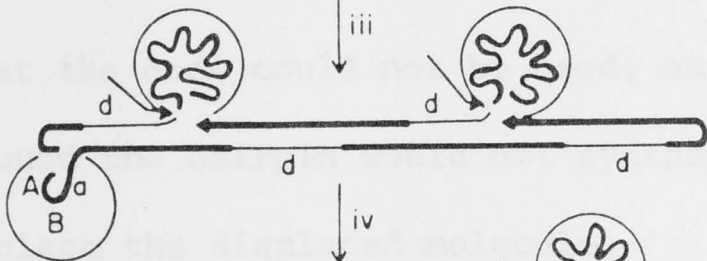
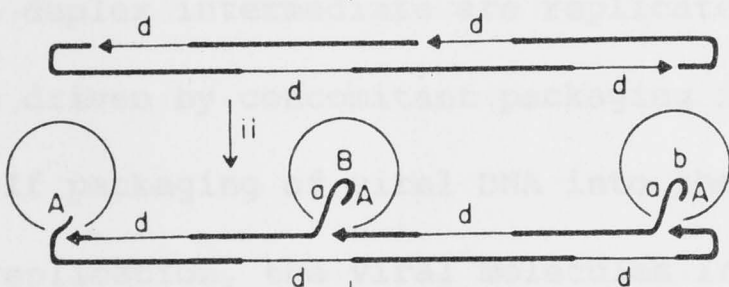
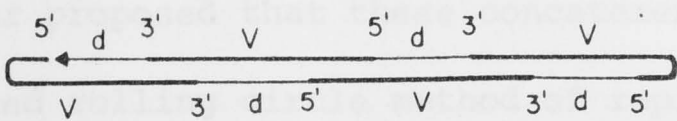
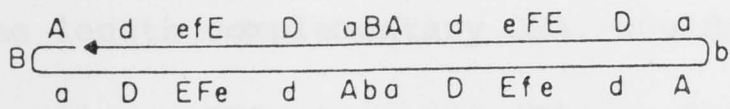
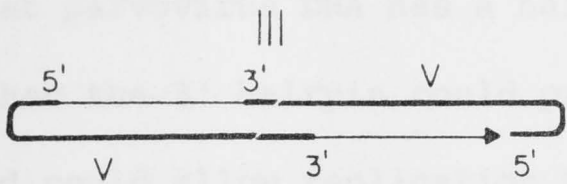
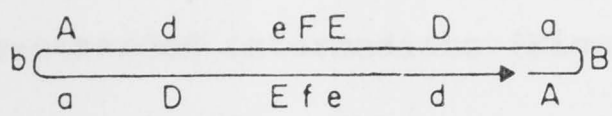
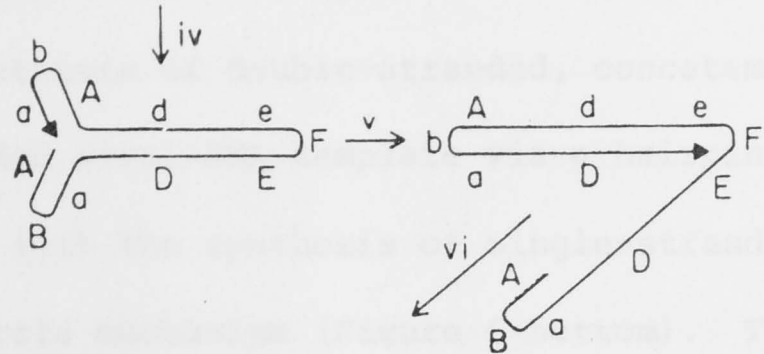
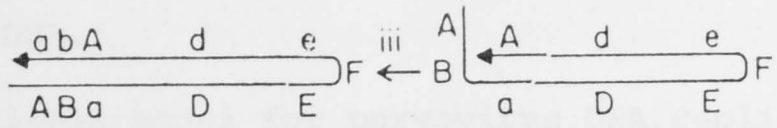
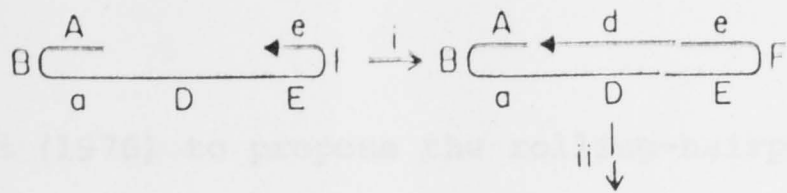
The rolling hairpin model for replication of non-defective parvovirus linear DNA.

Top: The hairpin-primed mechanism of producing concatemers of + and - strands. The viral (+) strand is represented by sequence ABaDEFe where sequence 'A' is complementary to 'a', etc. The solid triangle represents the 3' end of the DNA strand and direction of DNA replication. The hairpin on the 3' end of the viral strand primes synthesis of the complementary (-) strand. After replication of the 5' sequence (ABa), the 5' hairpin reforms and the newly replicated 3' hairpin primes DNA replication back along the viral strand, producing concatemers of alternating + (V, thick line) and partial - (thin line) strands. The process can be repeated to produce longer concatemers.

Bottom: Synthesis of new viral strands in the concatemer could displace the existing viral (+) strand (thick line, sequence V). This process could occur with concomitant packaging of the displaced viral (+) strand. An endonuclease (indicated by the arrows in step IV) would release the displaced viral (+) strand from the concatemer. A similar mechanism could be proposed for the defective parvovirus subgroup, with an inverted terminal repetition (sequence  $5' \text{ABa} 3'$  equal to sequence  $5' \text{EFe} 3'$ ) that would enable packaging of both (+) and (-) strands.

Taken from Tattersall and Ward (1976).





Tattersall and Ward (1976) to propose the rolling-hairpin mechanism for replication of the DNA.

The rolling hairpin model for parvovirus DNA replication is in two stages: (i) the synthesis of double-stranded, concatemeric DNA molecules from a single-stranded viral DNA template via a hairpin-primed mechanism (Figure 6 top), and (ii) the synthesis of single-stranded viral DNA by a modified rolling circle mechanism (Figure 6 bottom). The first step of synthesis of the double-stranded intermediate (Figure 6 top) takes advantage of the fact that parvovirus DNA has a hairpin structure on each end of the molecule so that the 3' hairpin could prime DNA synthesis and the hairpin on the 5' end could allow replication to proceed back along the viral strand. In this way, concatemers of viral DNA, which are linked by shorter than genome length complementary DNA, could be produced and be maintained in a duplex linear DNA structure (Figure 6 top). Tattersall and Ward (1976) further proposed that these concatemeric structures are then used for a modified rolling circle method of replication in which the viral strands in the duplex intermediate are replicated out of the concatemer, possibly driven by concomitant packaging into the virion (Figure 6 bottom). If packaging of viral DNA into the virion did drive this last stage of replication, the viral molecules in the concatemer that form a hairpin at the ends could not be used, as shown in Figure 6, since replication around the hairpin would not synthesize a full length viral molecule to replace the displaced molecule.

The model in Figure 6 shows complete synthesis and packaging of only viral (+) strands, which occurs for the autonomously replicating parvoviruses; however, both viral plus and minus strands could be synthesized by this mechanism because of the existence of an inverted terminal repetition in the AAV DNA. Packaging of both strands of AAV DNA in separate virion particles would fit in with recognition of a specific

terminal sequence being involved with packaging, as proposed by the model. Tattersall and Ward (1976) also proposed a hairpin-primed mechanism for replication of linear chromosomes such as eukaryote chromosomes and other linear DNA virus chromosomes that have terminal hairpin structures.

There is considerable evidence for a hairpin-primed mechanism for DNA replication of parvovirus DNA, which primarily is based upon the structure of the ends of the DNA and the existence of concatemeric structures. Most of the evidence comes from work on the adeno-associated subgroup of parvoviruses.

The structure of AAV DNA is complex. In a population of viral DNA molecules, the plus and minus strands isolated from the virions anneal to form linear, double-stranded monomers and dimers and also double-stranded circles (Gerry et al., 1973; Berns, 1974). The existence of double-stranded circles suggested some form of terminal repetition, as in phage T7. Koczot et al. (1973) and Berns and Kelly (1974) demonstrated that AAV DNA also had some form of inverted terminal repetition, similar to that found in adenoviruses (section 1.6), which caused formation of single-stranded circles with a duplex DNA projection (panhandle structure). An incorrect structure of the ends of the DNA was suggested from these studies (Gerry et al., 1973; Berns, 1974) and it was not until detailed restriction enzyme analysis of the termini was done, that correct structures were obtained. The unusual properties of the DNA were caused by complex secondary structure of the ends of the molecules which could rapidly form double-stranded (hairpin-like) structures after denaturation (Denhardt et al., 1976; Fife et al., 1977; de la Maza and Carter, 1977). This strongly suggested some form of terminal palindrome at each end of the molecule. The inverted terminal repetition has now been sequenced (Berns and Hauswirth, 1978) and the data are more consistent with two possible sequences at the ends, which result from the fact that the first 126



nucleotides (the terminal repetition) can exist in either of two orientations, 1 to 126 and 126 to 1. They termed this sequence a 'flip-flop' region which forms a complex hairpin structure, and its existence is suggestive evidence for hairpin priming for initiation of DNA replication.

Straus et al. (1976) and Handa et al. (1976) first observed concatemers of alternating plus and minus strands of AAV DNA as intermediates in DNA replication and proposed that initiation of DNA replication was primed by the 3'-hydroxyl provided by a hairpin at the 3' end of the molecule. Hauswirth and Berns (1977) demonstrated that the origin and terminus of replication in duplex AAV DNA were near the ends of the linear DNA, which is consistent with hairpin priming. Handa and Shimojo (1977) reported that the fast sedimenting concatemers could be converted to unit length molecules by digestion with protease and suggested a protein-linking molecule. However, the concatemers reported by Straus et al. (1976) were observed even when the DNA was isolated in the presence of protease and Lavelle and Li (1977) found no evidence of protein associated with the DNA after extraction in guanidine hydrochloride. This point has not been resolved, but perhaps Handa and Shimojo (1977) were observing fast sedimenting monomers that were partially packaged into the virion.

The non-defective parvoviruses that do not require a helper virus appear to replicate by a similar mechanism, even though they do not contain a detectable inverted terminal repetition and only package the viral plus strand into the virion (Tattersall and Ward, 1976). However, minute virus of mice (MVM) and Kilham rat virus (KRV) have a 5' terminal hairpin, as shown in Figure 6 (top) (Bourguignon et al. 1976; Salzman, 1977), and these viruses and other non-defective parvoviruses have a 3' terminal hairpin similar in structure to the AAV hairpin structure



(Bourguignon et al., 1976; Salzman, 1976; Rhode, 1977; Astell, 1978).

Both in vivo (Gunther and May, 1976; Rhode, 1977; Lavelle and Li, 1977) and in vitro studies (Salzman et al., 1978) have demonstrated concatemers of plus and minus strands of replicating non-defective parvovirus DNA and the data are consistent with the first part of the rolling hairpin model (Figure 6 top). Furthermore, the DNA is self-priming in an in vitro replication system with *E.coli* DNA polymerase (Salzman et al., 1978).

This evidence is good suggestive evidence for a mechanism which generates duplex concatemers as proposed by Tattersall and Ward (1976) (Figure 6 top). However, there is little evidence for the second stage of replication (Figure 6 bottom), except that it does explain the link between the inverted terminal repetition in AAV and packaging of both strands. Tattersall et al. (1973) have observed duplex molecules with single-stranded side chains in infected cells, which are consistent with the rolling hairpin model.

Hairpin-primed replication has been suggested for replication of the ends of linear adenovirus DNA (Wu et al., 1977; R.J. Roberts, personal communication to A.J.D. Bellett). I have examined this model for human adenovirus type 5 DNA replication (see Chapter 3).

### 1.5 Nucleic acid-protein complexes

In this section, I will discuss the interactions of proteins with nucleic acids in which at some stage a protein becomes covalently bound to the nucleic acid to form a complex. Some complexes of covalently bound DNA and protein have already been discussed in section 1.1 and include the covalent linkage of the  $\phi$ X174 cistron A protein to the 5' end of the nicked strand in replicating form DNA, DNA gyrase and the plasmid relaxation proteins which become covalently bound to the substrate DNA. However, several other bacteriophages and some eukaryotic

viruses also have protein covalently linked to their genome, which possibly have a function in DNA replication.

The nature of possible bonds between a nucleotide (or oligonucleotide) and amino acid (or peptide) was reviewed several years ago by Shabarova (1970). The *E.coli* DNA ligase enzyme has an adenylyl group covalently linked to the  $\epsilon$ -amino group of a lysine residue in the enzyme, as the first intermediate in the ligation of a nick in DNA (Gumport and Lehman, 1971). This linkage is a phosphoamide bond.

The bacteriophage  $\phi 29$  from *Bacillus subtilis* has a linear double-stranded DNA chromosome of molecular weight  $12 \times 10^6$  which can be isolated from virions as a DNA-protein complex (Ortin et al., 1971). The protein causes the DNA to circularize by non-covalent protein-protein interactions (Ortin et al., 1971; Hirokawa, 1972), and is linked to the 5' end (Yehle, 1978; Salas, et al., 1978) of the terminal restriction endonuclease fragments, as a result of which, these do not enter agarose gels when restricted DNA is subjected to electrophoresis (Streeck et al., 1974; Inciarte et al., 1976; Ito et al., 1976). The protein has been purified and shown to have a molecular weight of 28,000 to 31,000 (Yehle, 1978; Harding et al., 1978) and is the same as, or very similar to, the protein specified by  $\phi 29$  gene 3 (Salas et al., 1978; Harding et al., 1978). Since the protein is not removed from the DNA by treatment with agents such as phenol, SDS, urea, guanidine hydrochloride or sodium perchlorate (Hirokawa, 1972; Ito et al., 1976; Harding and Ito, 1976), it has been concluded that the protein is covalently linked to the DNA.

The protein found on the termini of virion DNA is also found on intracellular DNA (Harding and Ito, 1976) and also on DNA isolated from cells infected with a mutant  $\phi 29$  phage which does not make late proteins, suggesting that the protein is made early in infection (Harding and Ito, 1976). In addition, Hirokawa (1972) demonstrated that protease-treated

Ø29 DNA could not transfect cells, whereas phenol-extracted DNA-protein complex could. Yanofsky et al. (1976) have shown that DNA-protein complex isolated from a temperature-sensitive mutant in gene 3 was temperature-sensitive for transfection. The link between the gene 3 protein and the terminal protein of Ø29 (Yanofsky et al., 1976; Salas et al., 1978; Harding et al., 1978) has interesting implications as gene 3 mutants are defective in DNA replication, probably at the initiation step (McGuire et al., 1977).

Other small *Bacillus subtilis* phages also have protein firmly attached to their DNA. Phages GA-1 (Artwert and Venema, 1974; Arnberg et al., 1974; Arnberg and Artwert, 1976), Ø15 (Ito et al., 1976) and M2 (Kawamura and Ito, 1977) have protein linked to the termini, and for GA-1 and M2 phages, the ability of DNA to transfect cells is dependent upon the presence of the terminal protein (Artwert and Venema, 1974; Kawamura and Ito, 1976).

The DNA from adenoviruses is normally isolated as a linear double-stranded chromosome (section 1.6). In considering the problem of replication at the 5' ends of linear adenovirus DNA, Robinson et al. (1973) tested for a protein linked to the ends of the DNA, or origin of replication, as had been found in Ø29 or the ColE1 relaxation complex respectively. When DNA was isolated from virions by a number of methods which avoided protease digestion, including disruption in urea and sodium perchlorate, guanidine hydrochloride, sodium deoxycholate, formamide or pyridine, followed by extraction with chloroform-isoamyl alcohol or phenol, a protein was found on the ends of the DNA which caused the DNA to circularize when viewed in the electron microscope (Robinson et al., 1973; Robinson and Bellett, 1974; Robinson, 1974). The protein also caused the formation of oligomeric molecules which were sensitive to proteolytic enzymes, indicating that the end-to-end interactions were not



the result of covalent link of the two DNA molecules. The DNA-protein complex had a slightly lower buoyant density in CsCl gradients containing guanidine hydrochloride (GuHCl) and sedimented slightly faster than linear DNA in sucrose gradients containing GuHCl (Robinson et al., 1973; Robinson and Bellett, 1974). The fact that the DNA-protein complex was stable to protein denaturants suggested a covalent association of the terminal protein.

Subsequently, Rekosh et al. (1977) further characterized the protein and showed that it was linked to each of the 5' ends of the DNA. The DNA-protein complex was labeled in vitro with  $^{125}\text{I}$  using the Bolton and Hunter reagent and after digestion with deoxyribonuclease the protein moiety was shown to have an apparent molecular weight of 55,000. However, there were still some nucleotides attached to the protein, even after four hours of deoxyribonuclease treatment, and these were shown to consist only of deoxyadenosine, deoxythymidine and deoxycytidine monophosphates. The absence of deoxyguanine monophosphate was probably due to the absence of a G residue in the terminal 24 bases at the 5' end of DNA from adenovirus types 2 and 5 (Steenbergh et al., 1978; J.R. Arrand and R.J. Roberts, personal communication) and therefore sets a limit on how much 5' specific DNA remains on the protein. It also raises the possibility that up to 24 bases are still linked to the terminal protein and that the molecular weight of the protein is slightly less than 55,000.

Carusi (1977) has shown that adenovirus DNA-protein complex is resistant to digestion with the 5' specific  $\lambda$  exonuclease and that the 5' ends of the DNA were inaccessible to phosphorylation by polynucleotide kinase, even after treatment with bacterial alkaline phosphatase to remove any phosphate group. The DNA still had the 5' blocking group after protease digestion which confirms the covalent association of a peptide to the 5' terminal deoxycytidine. In addition, the 3' termini of adenovirus DNA



and DNA-protein complex are accessible to the 3' specific exonuclease III (Green et al., 1967; Youngusband and Bellett, 1971; Carusi, 1977; Estes, 1978), which again suggests that the protein is linked to the 5' termini of the linear DNA molecule.

The protein on the terminal restriction fragments of adenovirus DNA prevents those fragments from entering agarose gels when the restricted DNA is subjected to electrophoresis (Brown et al., 1975; Sharp et al., 1976; Padmanabhan and Padmanabhan, 1977; Estes, 1978). The protein can also be visualized in the electron microscope when it is complexed to other proteins (Keegstra et al., 1977; Wu and Davidson, 1978), but not when the DNA-protein complex alone is visualized in the electron microscope (Robinson et al., 1973). The technique of Wu and Davidson (1978) is a general one for nucleic acid-protein complexes. They treat the protein moiety with dinitrofluorobenzene and the resulting dinitrophenyl (DNP) groups are then complexed with anti-DNP IgG. The protein-(DNP)<sub>n</sub> (IgG)<sub>m</sub> complex (where n and m are the number of DNP or IgG molecules respectively) can then be observed in the electron microscope as a large complex on the nucleic acid strand.

The function of the adenovirus terminal protein is not known. Brown et al. (1975) suggested that the viral DNA is a covalently closed circle inside the virion and that upon isolation, the terminal protein acts as an endonuclease and attaches to the ends of the DNA. This was also considered by Robinson et al. (1973), but they considered this possibility unlikely. A covalently closed circular molecule as an intermediate in DNA replication, that is nicked by an endonuclease which becomes attached to the DNA, has been proposed (Robinson et al., 1973; Robinson and Bellett, 1974) but is now unlikely. Cordon et al. (1975) also proposed a circular form of a DNA-protein complex that is packaged inside the icosahedral adeno-virion.

Adenovirus DNA is not very infectious and is normally precipitated with calcium phosphate in infectivity assays (Graham and van der Eb, 1973). The infectivity of the DNA-protein complex is about  $10^2$  to  $10^3$  fold higher than deproteinized DNA when assayed by this technique (Sharp et al., 1976; Chinnadurai et al., 1978) and Sharp et al. (1976) suggested that one possible function of the protein is to protect the DNA from nuclease attack, especially in the artificial infectivity assay. Functions for the adenovirus terminal protein in viral DNA replication have also been suggested and will be discussed further in Chapter 6.

Kasamatsu and Wu (1976a; 1976b) have reported the association of a protein or protein complex at the origin of simian virus 40 DNA replication. The protein(s) are associated with one strand at a nick in that strand that is induced by isolating the DNA-protein complex in the presence of dithiothreitol, the other strand being covalently closed. The nick can occur on either strand in a staggered site, each site being a few hundred nucleotides apart (Kasamatsu and Wu, 1976b). As the complex is stable in alkali and protein denaturants, they concluded the protein(s) is covalently bound. This could represent a similar situation to the relaxation proteins found at the origins of replication of bacterial plasmids (Helinski, 1976).

Proteins have been found covalently linked to the ends of the genome of some RNA viruses of eukaryotes, including poliovirus RNA (Flanegan et al. 1977; Lee et al., 1977; Nomoto et al., 1977) and foot-and-mouth disease virus RNA (Sanger et al., 1977). The poliovirus terminal protein (VPg) has a molecular weight of 12,000 daltons and is linked to the 5' termini of virion RNA and replicating intermediate RNA by a phosphodiester bond between a tyrosine residue and the 5' terminal uridine (Ambros and Baltimore, 1978; Rothberg et al., 1978). The protein is also linked to short nascent RNA strands, isolated from replicative intermediate RNA

(Pettersson et al., 1978). The protein has been implicated in RNA replication (Nomoto et al., 1977; Flanagan et al., 1977; Pettersson et al., 1978), possibly by the VPg-pU complex acting as a primer for a primer-dependent RNA polymerase (Flanagan et al., 1977).

A firm association of a short RNA moiety approximately 350 nucleotides long has been reported for ribonuclease P from *E. coli* which is necessary in the biosynthesis of the 5' termini of transfer RNA molecules (Stark et al., 1978). It is possible that this RNA component is involved in recognizing the substrate for ribonuclease activity (Stark et al., 1978).

#### 1.6 Adenovirus DNA and DNA replication

Adenoviruses (Ad) were first discovered in 1953 as cytopathogenic agents from degenerating human adenoid tissue and were first named adenoviruses in 1956 to indicate the group of viruses isolated from the respiratory tracts of man and animals. In humans they primarily cause mild respiratory disease, but can also cause, among other diseases, keratoconjunctivitis, acute infections of the upper respiratory tract and bronchial pneumonia (Sohier et al., 1965; Philipson et al., 1975). They were also identified as the first oncogenic virus of human origin when they were shown to cause undifferentiated sarcomatous tumors at the site of inoculation into hamsters (Sohier et al., 1965; Green, 1970). The human adenoviruses have been divided into groups on the basis of their oncogenicity in newborn hamsters (Huebner, 1967; Green, 1970; Philipson et al., 1975). Subgroups A, B and C are respectively highly, moderately and non-oncogenic in this system.

The adenoviruses are non-enveloped viruses with an icosahedral virion structure, primarily made up of hexon, penton and fiber proteins. Inside the particle is a core, consisting of DNA and core proteins, mainly proteins V and VII. For a review on the structure and composition of the



virion and the adenovirus proteins, see Philipson et al. (1975).

The adenovirus genome is a linear double-stranded DNA molecule with a molecular weight of 20 to 23 x 10<sup>6</sup> for different human serotypes and as high as 29 x 10<sup>6</sup> for an avian adenovirus (Green et al., 1967; van der Eb et al., 1969; Younghusband and Bellett, 1971). Partial denaturation maps of adenovirus DNA show that adenovirus genome is unique and not circularly permuted (Doerfler and Kleinschmidt, 1970; Doerfler et al., 1972; Younghusband and Bellett, 1972; Ellens et al., 1974), but that the maps for the closely related serotypes Ad2 and Ad5, are similar. Analysis of the termini of adenovirus DNA also indicates that the genome is not circularly permuted (Murray and Green, 1973). The ends of the molecule have an inverted terminal repeated sequence (Garon et al., 1972; Wolfson and Dressler, 1972) which does not allow double-strand circle formation after digestion with exonuclease III and annealing (Green et al., 1967; Younghusband and Bellett, 1971; Chapter 2, this thesis), but allows formation of single-strand circles when the DNA is denatured and reannealed at low concentration or after separation of the complementary strands. The length of the inverted terminal repetition was estimated to be 100 to 140 base pairs in length (Roberts et al., 1974; Robinson and Bellett, 1975) and single-stranded circles were due to hydrogen bonding that could be eliminated by digestion with exonuclease III (Wolfson and Dressler, 1972; Robinson and Bellett, 1975). The inverted terminal repetition has subsequently been sequenced for adenovirus type 5 (Ad5) and adenovirus type 2 (Ad2) (Steenbergh et al., 1978; R.J. Roberts and J.R. Arrand, personal communication), giving a repetition of 102 base pairs for Ad2 and 103 base pairs for Ad5, the only difference between the sequence in the two serotypes being an A/T insertion in the Ad5 sequence compared with the Ad2 sequence.



As described in section 1.5, adenovirus DNA can be isolated from the virion as a DNA-protein complex which consists of the linear DNA with a 55,000 dalton protein covalently attached to the 5' ends of the molecule (Robinson et al., 1973; Robinson and Bellett, 1974; Rekosh et al., 1977; Carusi, 1977). The DNA exists in the virion in a core structure which is very similar to the structure of eukaryotic chromatin (Brown et al., 1975; Corden et al., 1976). A review of the structure of adenovirus DNA and homology of DNA between serotypes can be found in Philipson et al. (1975).

A standard nomenclature for the adenovirus genome has been accepted (Bachenheimer et al., 1977) in which the linear DNA is drawn so that the region coding for the 'early' transformation genes is on the left end. The r strand of the DNA codes for mRNA which is synthesized from right to left as the map is drawn (5' to 3' direction) and leftward transcription occurs on the l strand of the DNA (Figure 8). Thus a standard order of genes on the DNA is obtained.

Adenovirus DNA replication starts in the cell nucleus about 7 to 10 hours post infection in spinner cultures of human cells, depending upon the multiplicity of infection (Ginsberg et al. 1967). However, replication can start at a later stage with low multiplicity of infection of monolayers of human cells (see Chapter 4). Host cell DNA replication is shut off after viral DNA replication begins, and in spinner cultures at high multiplicity of infection, viral DNA replication reaches a maximal rate at 13 to 14 hr post infection and begins to decline at 16 to 22 hr post infection (Ginsberg et al., 1967; Pina and Green, 1969).

Replication of adenovirus DNA proceeds in the 5' to 3' direction on each strand with origins and termini of replication at or near each end of the linear molecule. This was determined by using the replication mapping method developed by Danna and Nathans (1972). Viral DNA was

pulse-labeled for a short period, molecules that had completed replication during the short pulse were selected by chromatography on benzoylated, naphthoylated DEAE cellulose and analysed by restriction enzyme techniques. The termini of replication are located at the 3' end of each strand (Schilling et al., 1975; Tolun and Pettersson, 1975; Horwitz, 1976; Weingärtner et al., 1976; Ariga and Shimojo, 1977; Sussenbach and Kuijk, 1977; Arens and Yamashita, 1978) and the origins at the 5' end of each strand (Horwitz, 1976; Ariga and Shimojo, 1977; Sussenbach and Kuijk, 1978). Analysis of pulse-labeled adenovirus-SV40 hybrids (Horwitz, 1974) and electron microscopy of adenovirus DNA (see below) also support these conclusions. Sussenbach and Kuijk (1978) have shown that the origin of replication is located within 70 base pairs of the ends of the molecule, and it is likely that the origins are at the very ends of the molecule.

Ariga and Shimojo (1978) have reported that the origin of DNA replication is a short distance (6 to 20 base pairs) from the end of the molecule. Their results were based upon failure to detect labeled oligonucleotides from the 5' end of pulse-labeled DNA. However, the equivalent class of oligonucleotides which was detected in uniformly labeled DNA was sensitive to alkaline phosphatase. This is inconsistent with reports that intracellular viral DNA (Estes, 1978) and virion DNA (Carusi, 1977) have a blocking group other than a free phosphate on each 5' end, causing the DNA to be resistant to alkaline phosphatase and  $\lambda$  exonuclease. The blocking group is probably a fragment of the terminal protein. I also doubt whether there was sufficient radioactivity in pulse-labeled DNA to detect short oligonucleotides from the 5' end by this method. Whether the exact location of the origin of DNA replication is within the terminal 70 base pairs or at the very end of the molecule is still not known.

The basic mechanism of adenovirus DNA replication has been reviewed previously (Levine et al., 1976). Replicating adenovirus DNA that has been pulse-labeled with  $^3\text{H}$ -thymidine sediments in neutral sucrose gradients more rapidly than completed (or virion) DNA (Pearson and Hanawalt, 1971; Bellett and Youngusband, 1972; Sussenbach and van der Vliet, 1972; van der Eb, 1973; Bourgaux-Ramoisy et al., 1974) and has a higher buoyant density in  $\text{CsCl}$  gradients than completed (or virion) DNA (Pearson and Hanawalt, 1971; Bellett and Youngusband, 1972; Sussenbach and van der Vliet, 1972; van der Eb, 1973; Pettersson, 1973; Pearson, 1975). These results were due to the high percentage (approximately 20-30%) of single-stranded DNA in the replicating pool, as detected by chromatography on benzoylated naphthoylated DEAE cellulose (Bellett and Youngusband, 1972; van der Eb, 1973), sensitivity to  $\text{S1}$  nuclease (Pettersson, 1973; Pearson, 1975) or electron microscopy (Sussenbach et al., 1972; van der Eb, 1973; Ellens et al., 1974; Lechner and Kelly, 1977).

The nature of the single-stranded regions has been analysed by hybridization to separated strands of adenovirus DNA (Tolun and Pettersson, 1975; Lavelle et al., 1975; Flint et al., 1976; Sussenbach et al., 1976). Both strands were represented in the single-stranded fraction of replicating DNA, indicating asynchronous synthesis from either end of the molecule. In addition, completely single-stranded, genome-length molecules have been observed by electron microscopy (Sussenbach et al., 1972; van der Eb, 1973; Ellens et al., 1974; Lechner and Kelly, 1978), as well as branched intermediates with a single-stranded arm. These observations are consistent with a strand displacement mechanism for adenovirus DNA replication (Sussenbach et al., 1972; Ellens et al., 1974; Sussenbach et al., 1974) in which replication is initiated at either end of the molecule and proceeds unidirectionally, displacing the non-template strand as



single-stranded DNA (Lechner and Kelly, 1977; Kedinger et al., 1978).

The displaced strand is then replicated by initiation at the opposite end. The original model described by Sussenbach et al. (1974) proposed that initiation only occurred at the right hand end, but this is inconsistent with current data (Sussenbach et al., 1976). The current strand displacement mechanism of adenovirus DNA replication is shown in Figure 7.

The pulse-labeled replicating DNA sediments more slowly than mature DNA in alkaline sucrose gradients (Horwitz, 1971; Pearson and Hanawalt, 1971; Bellett and Younghusband, 1972; Sussenbach and van der Vliet, 1972; Pettersson, 1973; van der Eb, 1973) and covalently closed circular intermediates have not been found (Bellett and Younghusband, 1972; Doerfler et al., 1973). This would rule out closed circle, rolling circle or concatemeric intermediates in adenovirus DNA replication.

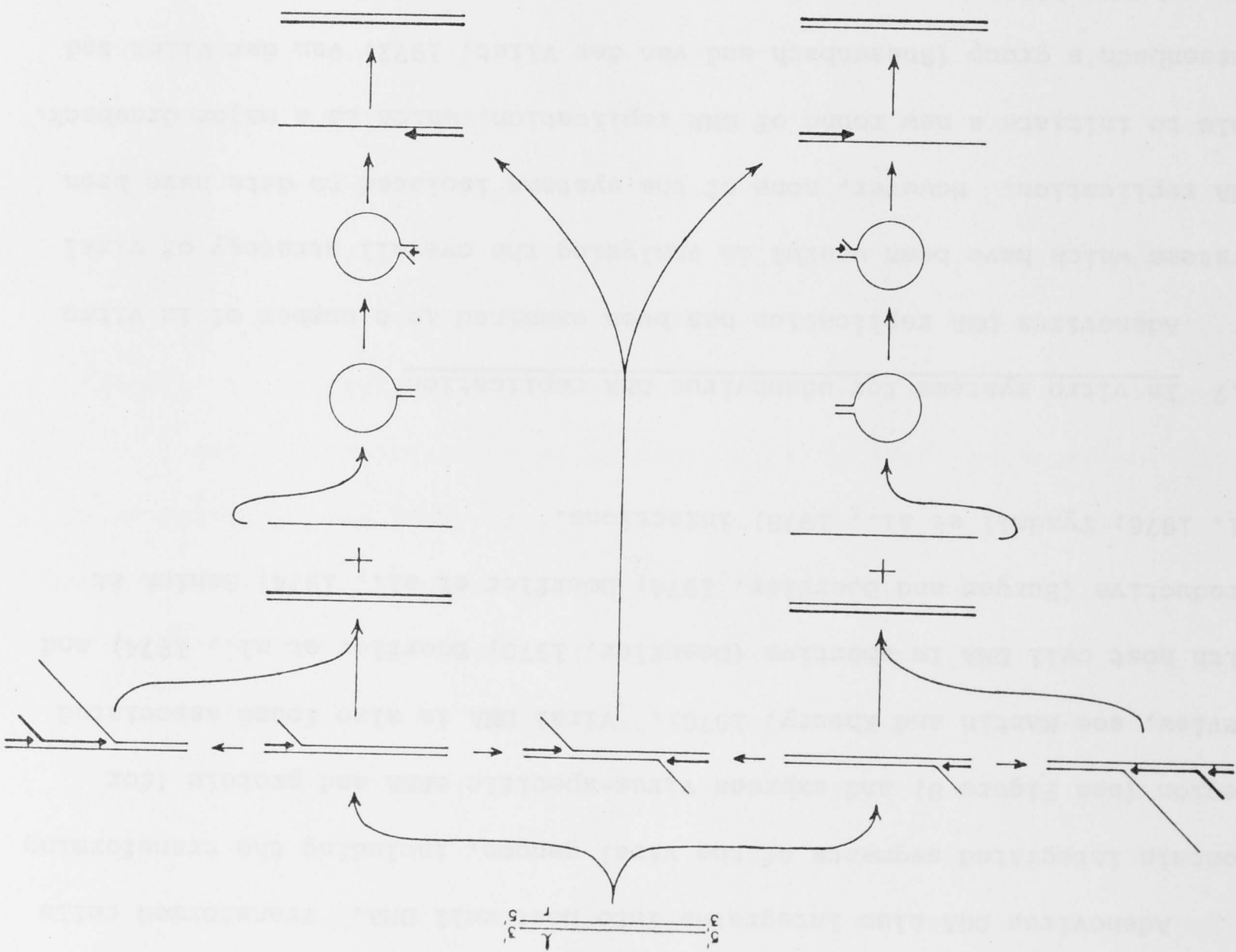
As indicated in Figure 7, adenovirus DNA replication is semi-conservative (Bellett and Younghusband, 1972; van der Vliet and Sussenbach, 1972) and because elongation is only in the 5' to 3' direction (unidirectional at the fork), then there is no need for Okazaki-type fragments. However, Bellett and Younghusband (1972) and Winnacker (1975) have reported short DNA fragments that can be chased into mature DNA. Short fragments of DNA have also been shown to accumulate in the presence of hydroxyurea (Vlak et al., 1975; Winnacker, 1975). Due to the problems of uracil excision (section 1.1) causing short DNA fragments, their existence might not indicate discontinuous synthesis by Okazaki fragments and it will be necessary to demonstrate RNA linked to DNA as an intermediate in DNA replication, as has been done for papovavirus DNA replication, before any definite conclusions can be made. Of course, if Okazaki-like fragments were primed by deoxynucleotides, the RNA linked to DNA would not be detected.



Figure 7

A model for adenovirus DNA replication. Initiation of viral DNA replication occurs at either end (and less frequently at both ends) and proceeds in the 5' to 3' direction, displacing the non-template strand as a single strand. These replicating molecules have been designated form I molecules. The displaced single strands are replicated from the origin at the other end of the molecule and have been designated form II replicating molecules. It is possible that the displaced single strand circularizes by a 'panhandle' structure due to the inverted terminal repetition, and a double-stranded origin of replication could be maintained, but this has not been demonstrated. This model does not explain how the 5' ends of the newly replicated strand are synthesized (see text).

Taken from Lechner and Kelly (1977).



It was seen in sections 1.3 and 1.4 that a problem exists in the replication of the ends of linear chromosomes, as the known DNA polymerases require a free 3'-hydroxyl to act as a primer for replication. This problem exists in adenovirus DNA replication (Bellett and Younghusband, 1972) and is discussed in detail in Chapters 3 and 6.

Adenovirus DNA also integrates into host cell DNA. Transformed cells contain integrated segments of the viral genome, including the transforming region (see Figure 8) and express virus-specific mRNA and protein (for review, see Martin and Khoury, 1976). Viral DNA is also found associated with host cell DNA in abortive (Doerfler, 1970; Doerfler et al., 1974) and productive (Burger and Doerfler, 1974; Doerfler et al., 1974; Schick et al. 1976; Tyndall et al., 1978) infections.

#### 1.7 In vitro systems for adenovirus DNA replication

Adenovirus DNA replication has been examined in a number of in vitro systems which have been useful in analysing the overall strategy of viral DNA replication. However, none of the systems isolated to date have been able to initiate a new round of DNA replication, which is a major drawback. Sussenbach's group (Sussenbach and van der Vliet, 1972; van der Vliet and Sussenbach, 1972) have used isolated nuclei from adenovirus-infected cells and find that adenovirus replication proceeds in a similar way to that found in infected cells, except that the overall rate of elongation of replicating viral DNA that had been initiated in vivo was slower than the rate found in infected cells.

In vitro adenovirus DNA replication has also been reported in soluble extracts from infected cell nuclei. Yamashita et al. (1977) described a soluble replication system prepared from the nuclei of Ad2-infected human KB cells in which replicating viral DNA molecules (initiated in vivo) were elongated to form viral-length strands. Replication was semi-



conservative and exclusively in viral DNA molecules. The activity of an endogenous DNA polymerase was 10 to 34 times higher than in an equivalent extract from uninfected cells and the activity was increased by addition of sodium citrate. A number of enzyme activities in the soluble extract have been identified (Arens et al., 1977) including DNA polymerases  $\alpha$  and  $\gamma$ , RNA polymerase, DNA ligase and also the adenovirus-specific DNA-binding protein (see section 1.9).

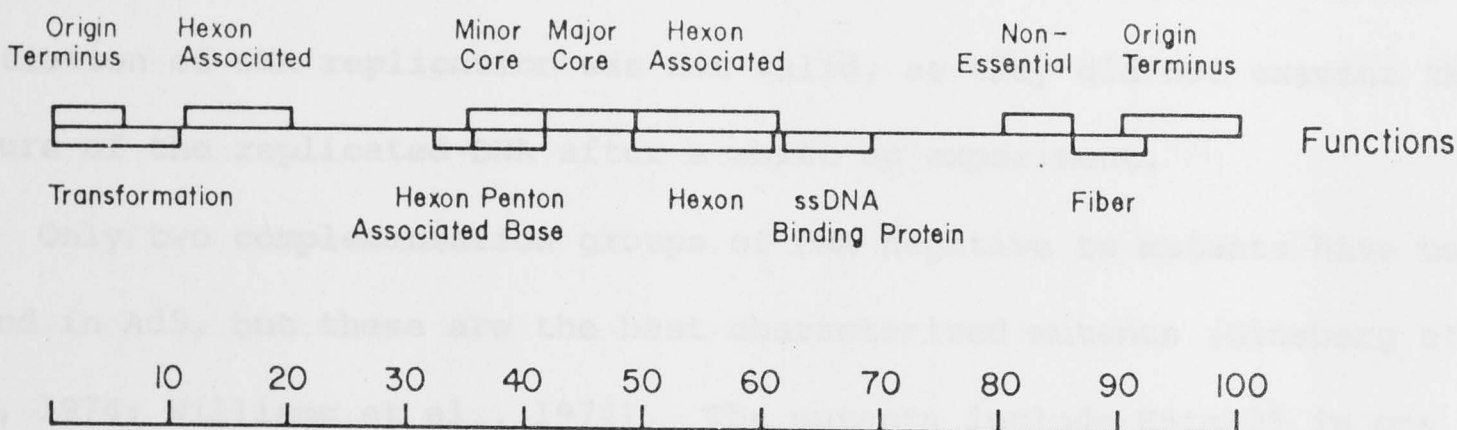
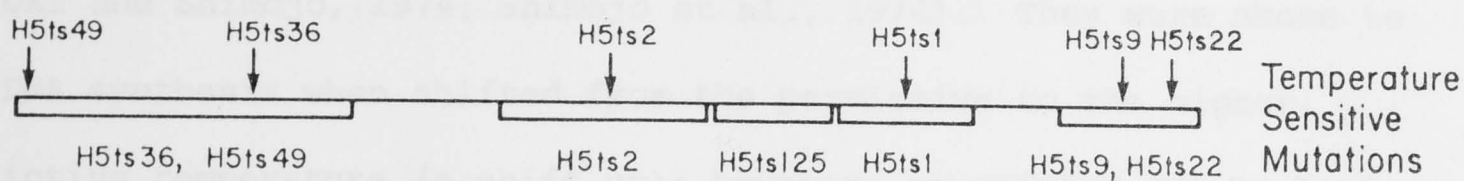
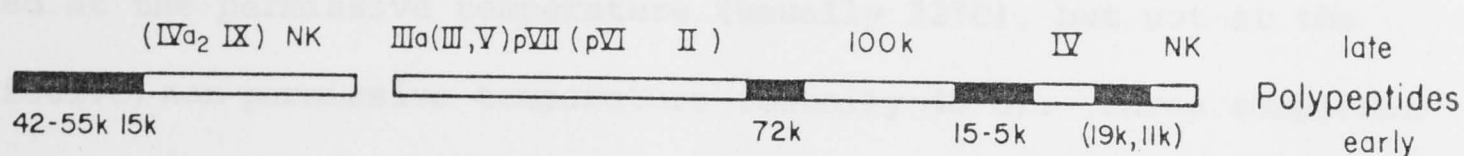
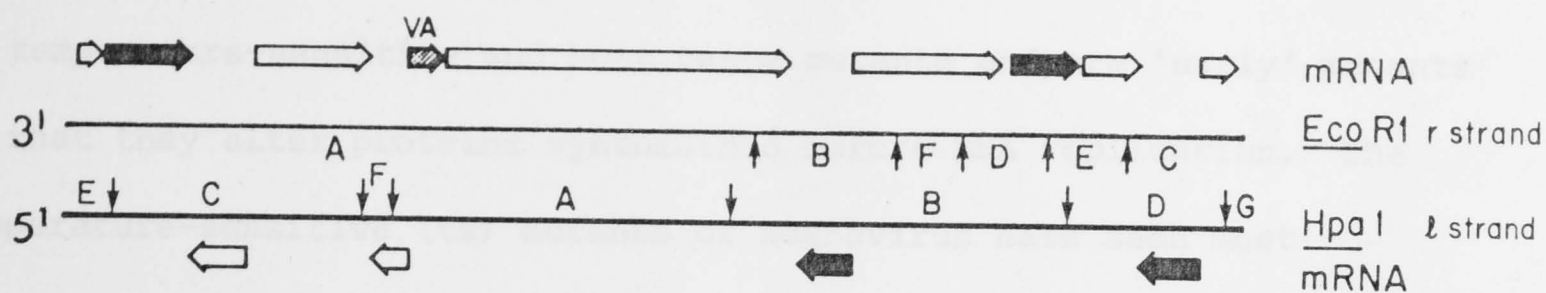
In vitro elongation of DNA replication has also been obtained in a membrane-associated complex (Yamashita and Green, 1975; Frenkel, 1978) in which viral DNA replication appears to be mediated by DNA polymerase  $\alpha$  (Frenkel, 1978). Recently, soluble replication systems have been described by Brison et al. (1977) and Kaplan et al. (1977) which were isolated from adenovirus-infected human HeLa cell nuclei in a similar way to the isolation of adenovirus transcriptional complexes (Wilhelm et al., 1976). The elongation and termination of viral DNA replication that had been initiated in vivo were essentially the same as in vivo replication. Both soluble extract systems were dependent upon  $Mg^{2+}$  and addition of all four deoxynucleotide triphosphates, but did not require additional ribonucleotide triphosphates (Yamashita et al., 1977; Brison et al., 1977; Kaplan et al., 1977). The soluble extract system of Kaplan et al. (1977) isolated from cells infected with a temperature-sensitive mutant of adenovirus (ts125), which has a temperature-sensitive DNA-binding protein (see below), maintains the temperature sensitivity of the protein in vitro if the extract is incubated at the non-permissive temperature prior to addition of the deoxynucleotide triphosphates (Horwitz, 1978). This system could be useful in defining protein and enzyme activities involved in the elongation adenovirus DNA replication.



Figure 8

A map of adenovirus DNA showing the restriction enzyme cleavage points for Ad2 DNA and mRNA transcripts of Ad2 and Ad5 DNAs. Early mRNA is indicated by the black blocks and late mRNA is indicated by the white blocks, with arrows on the 3' end of the mRNA. Some of the identified adenovirus proteins are shown, as well as the map position for some temperature-sensitive mutants of Ad5.

Taken from review by Flint (1977).



### 1.8 Mutants of adenovirus defective in viral DNA replication

Two classes of mutants affect replication of adenovirus DNA, classed as temperature-sensitive and host range mutants and are 'early' mutants in that they alter proteins synthesised before DNA replication. The temperature-sensitive (ts) mutants of adenovirus have been most characterized (Ginsberg and Young, 1976) and allow DNA replication to proceed at the permissive temperature (usually 32°C), but not at the restrictive, non-permissive temperature (usually 40°C). Three complementation groups of DNA negative ts mutants have been described in Ad12 (Shiroki and Shimojo, 1974; Shimojo et al., 1974). They were shown to stop DNA synthesis when shifted from the permissive to the higher, restrictive temperature (a shift up); however, as pointed out by Levine et al. (1976), their conclusion that all mutants were defective in the initiation of DNA replication was not valid, as they did not examine the nature of the replicated DNA after a shift up experiment.

Only two complementation groups of DNA negative ts mutants have been found in Ad5, but these are the best characterized mutants (Ginsberg et al., 1974; Williams et al., 1974). The mutants include H5ts125 in one complementation group and H5ts149, H5ts36 and H5ts37 in the other group; the H5 indicating that the virus is human adenovirus type 5. The H5ts125 mutant stopped synthesizing viral DNA when shifted to the non-permissive temperature, with a 90% reduction within one hour after the shift up, and the replicating DNA was converted into mature, completed molecules (van der Vliet and Sussenbach, 1975). From this it was concluded that the mutant was defective in initiation of replication, but this may not be so (see section 1.9). The H5ts125 mutation was shown to alter an adenovirus DNA-binding protein which binds specifically to single-stranded DNA (van der Vliet et al., 1975). The function of this protein is discussed in section 1.9. This mutant also has altered properties for



transformation of rat embryo cells (Ginsberg et al., 1974). At both the permissive and non-permissive temperatures, H5ts125 induced a 4-fold increase in the number of transformed cell foci of rat embryo cells when compared with wild-type virus. This could be due to the over-production of early proteins required for transformation; however, the increased ability to transform cells at the permissive temperature suggests a more complicated explanation. In addition, the H5ts125 mutation has been shown to have a host range effect, since viral DNA is not made at either the permissive or non-permissive temperature in mouse cells (Tyndall, 1978).

Adenovirus DNA negative mutants, H5ts36, H5ts149 and H5ts37 are in the same complementation group (Ginsberg et al., 1974; Williams et al., 1974) and the first two have been mapped in the left hand end of the genome, probably within the transforming region (see Figure 8). The function of the H5ts36 gene is not known, but a role in initiation of DNA replication has been suggested, based upon temperature shift experiments (van der Vliet and Sussenbach, 1975). After a shift up to the non-permissive temperature, H5ts36 DNA replication only begins to decline 6 hr later, while replication of H5ts149 begins to decline after only 1 to 2 hr (Ginsberg et al., 1974; van der Vliet and Sussenbach, 1975). Both these mutants fail to accumulate replicating molecules, suggesting a block in initiation of replication.

There is a difference in the transforming capability of these mutants which is still not explained. Ginsberg et al. (1974) reported that H5ts149 has similar properties to wild-type virus in the ability to induce foci on monolayers of rat embryo cells, whereas Williams et al. (1974) reported that H5ts36 and H5ts37 are defective in the initiation of transformation at the non-permissive temperature, but not at the permissive temperature. Unfortunately, this work was done in two separate laboratories and needs to be confirmed, but it might indicate a

dual function for that gene, one in transformation and the other in DNA replication. Williams et al. (1974) have demonstrated that H5ts36 and H5ts37 are not defective in the maintenance of transformation.

Three DNA negative complementation groups in adenovirus type 2, which is closely related to Ad5, have been described by Kathmann et al. (1976). One mutant, H5ts201, does not complement H5ts125 and is probably a DNA-binding protein mutant, and a second group, represented by H2ts206, complemented both H5ts36 and H5ts125. However, this mutant did not synthesize early viral messenger RNA and was temperature-sensitive in the first hour of infection and so is unlikely to be directly involved in DNA synthesis.

Recently, a series of host range (hr) mutants of adenovirus type 5 have been isolated and some have a DNA negative phenotype (Takahashi, 1972; Minekawa et al., 1976; Harrison et al., 1977). The mutants isolated by Harrison et al. (1977) have been best characterized for DNA synthesis and transformation. These mutants are able to grow on human embryo kidney cells that had been transformed with sheared Ad5 DNA, called 293 cells, but not on normal HeLa cells. They have been classified into two groups (I and II) on the basis of intergroup complementation and recombination and a number of properties characteristic of each group. Group I host range mutants are DNA negative in the non-permissive HeLa cells and do not synthesize detectable late proteins or the early DNA-binding protein (Lassam et al., 1978). They do not transform rat embryo or rat embryo brain cells, but abortively transform baby rat kidney cells with several times the efficiency of wild-type Ad5 (Graham et al., 1978). Group II mutants are DNA positive in HeLa cells and can also grow in normal human embryo kidney cells (HEK) the parent line of 293 cells (Harrison et al., 1977; Lassam et al., 1978). They synthesize the DNA-binding protein in HeLa cells and reduced amounts of late protein,

but are defective in the synthesis of an early 58,000 protein produced in 293 cells and Ad5-transformed cells (Lassam et al., 1978). The group II mutants are unable to transform any rat cell line tested, using a low calcium focus selection test (Graham et al., 1978). Thus it is clear that mutants of adenovirus that are defective in adenovirus DNA replication have altered abilities to transform cells. But not all transformation negative mutants have altered properties for viral DNA replication (e.g. group II host range mutants).

#### 1.9 Adenovirus single-stranded DNA-binding protein

The only characterized adenovirus-coded protein that is involved in viral DNA replication is the single-stranded DNA-binding protein. Two proteins were extracted from adenovirus-infected cells by chromatography on single strand DNA linked to cellulose. They were shown to have molecular weights of 72,000 and 48,000 respectively in Ad5 infected cells (van der Vliet and Levine, 1973) and 60,000 and 48,000 in Ad12 infected cells (Rosenwirth et al., 1975). The Ad5 or Ad2 DNA-binding proteins have been highly purified and shown to have a mol. wt. of 72,000 (72K) or 73,000 (73K) (Rosenwirth et al., 1976; Linné et al., 1977; Sugawara et al. 1977a). The molecular weight of the equivalent Ad12 protein was 58,000 (Rosenwirth et al., 1976). Tryptic peptide maps of the purified 72K and 48K proteins isolated from Ad5 infected cells showed similar peptide sequences in both proteins and indicated that the 48K protein was a cleavage product of the 72K protein (Rosenwirth et al., 1976). The Ad2 and Ad5 proteins have similar peptides, but are quite distinct from the Ad12 protein (Rosenwirth et al., 1976). The 72K DNA-binding protein is phosphorylated in vivo and can be phosphorylated in vitro (Jeng et al., 1977; Linné et al., 1977; Levinson et al., 1977; Russell and Blair, 1977); however, the 48K protein is not phosphorylated.



The 72K protein or DNA-binding protein is involved in viral DNA replication (van der Vliet and Sussenbach, 1975; van der Vliet et al., 1975) and van der Vliet et al. (1977) have presented evidence for a function in both initiation of viral DNA synthesis and elongation of nascent DNA chains. The role in elongation is probably to bind to the large amount of single-stranded DNA in adenovirus replicating DNA, as antibody against 72K protein inhibits chain elongation (van der Vliet et al., 1977) and the temperature-sensitive protein from H5ts125-infected cells is temperature-sensitive in both binding to single-stranded DNA and in viral DNA replication in vitro (Horwitz, 1978). A role in initiation of DNA replication has been proposed (van der Vliet and Sussenbach, 1975; van der Vliet et al., 1977) based upon in vivo temperature shift experiments with H5ts125-infected cells. However, Horwitz (1978) recently demonstrated that in an in vitro replication system containing the temperature-sensitive 72K protein, elongation does not occur at the non-permissive temperature after a shift from the permissive temperature, provided that the DNA precursor deoxynucleotide triphosphates were added after the shift up. This temperature sensitivity could be reversed by addition of wild type 72K protein to the in vitro reaction. If temperature sensitivity of the 72K DNA-binding protein in H5ts125 infected cells requires pre-incubation at the non-permissive temperature in vivo, similar to that shown by Horwitz (1978) for in vitro DNA replication, then an elongation defect would not have been detected by van der Vliet and Sussenbach (1975). Thus their interpretation that the H5ts125 mutant is defective in initiation and not in elongation needs reappraisal. Kedinger et al. (1978) have observed in the electron microscope, form I and form II (see Figure 7) molecules with the DNA-binding protein protecting single-stranded regions of replicating DNA.

The adenovirus DNA-binding protein is coded by a gene mapping at 62.4% to 67.1% from the left hand end of the DNA and is transcribed from the 1 strand (Figure 8) (Chow et al., 1977). A temperature-sensitive mutant, H5ts125 (section 1.8) maps within this region (Figure 8) and has an altered DNA-binding protein (van der Vliet and Sussenbach, 1975). The 72K protein is expressed in both productively infected cells and some adenovirus-transformed cells (Gilead et al., 1976a; Levinson et al., 1976; Sugawara et al., 1977b) and its synthesis in productively infected cells peaks at the same time as the peak of DNA synthesis (Gilead et al., 1976; Sugawara et al., 1977b).

A possible role for regulation by the 72K early protein has been proposed by Carter and Blanton (1978). They demonstrated that in cells infected with H5ts125 at the non-permissive temperature, early mRNA was over produced when compared with wild-type virus. Ginsberg et al. (1978) have shown that the 72K protein, made at the restrictive temperature, is degraded and is not immunologically reactive, whereas wild-type protein is produced and reactive at the non-permissive temperature.

It is beyond the scope of this review to include the vast amount of work on adenovirus transcription and protein synthesis. However, general reviews have been published (Philipson et al., 1975; Flint, 1977; Chow et al., 1977). A map of adenovirus DNA showing early and late cytoplasmic transcripts, polypeptides and adenovirus mutants is shown in Figure 8.

#### 1.10 Stimulation of host cell DNA replication and its relationship to transformation by DNA tumor viruses

The oncogenic DNA tumor viruses can induce tumors in rodents and the cell culture equivalent to this is a process called cell transformation. Transformation is a general term for the altered cell properties that are induced by these viruses, and can include induction of proteolytic enzymes,

changes in cell morphology, alteration in cellular growth behaviour such as increased saturation density, reduced serum requirement, decreased doubling time and anchorage independent growth in semi-solid media, modified cell surface components and stable expression of viral antigens (tumor or T-antigens) (see Green, 1970; Gallimore et al., 1977). Whether any of these characteristics of transformed cells correlates with in vivo tumorigenicity when the cells are injected into recipient animals is still confusing (Shields, 1976). We have seen previously that some adenovirus mutants defective for viral DNA replication are also defective for transformation, and this is also true for SV40 and polyoma mutants (see Weinberg, 1977 for a review). The oncogenic DNA viruses also induce host cell DNA replication after infection (Green, 1970; Strohl, 1973; Weil et al., 1974) and this probably plays an important role in the events leading to cell transformation and tumorigenesis. These observations link the mechanism of initiation of viral and cell DNA replication with the mechanism of cell transformation, all of which may be controlled by early viral gene expression.

Rat cells transformed by human adenovirus have been selected in low calcium (0.1mM) media as foci of epitheloid cells overgrowing the cell monolayer (McAllister et al., 1969). Transformed cells selected by this procedure have two stable characteristics. They have continued expression of the virus induced tumor antigen in a proportion of the cells (McDougall et al., 1974; Gallimore, 1974; McDougall et al., 1977; Gallimore et al., 1977) and retain part of the viral genome covalently integrated into the host cell DNA (Gallimore et al., 1974; Sambrook et al., 1974; Bellett, 1975; Green et al., 1976; Groneberg et al., 1977). In human adenovirus type 2 transformed rat cells, the left hand 14% of the viral DNA, or the transforming region (Figure 8) is always present (Sambrook et al., 1974; Gallimore et al., 1974) and this early gene region is preferentially



transcribed in transformed cells (Sharp et al., 1974; Flint and Sharp, 1976; Sharp and Flint, 1976). This region contains some of the DNA negative, transformation negative mutants discussed in section 1.8.

Another group of DNA tumor viruses, the papovavirus group which includes SV40 and polyoma virus, can transform cells and the transformed cells have been well characterized. SV40 and polyoma virus transformed cells have been selected as either foci of cells in liquid media or as colonies of cells growing in semi-solid media (see Rassoulzadegan et al., 1978). The stability of the transformed phenotype relative to expression of the viral tumor antigen depends upon the method of selecting the transformed cells, as discussed below.

Whilst trying to obtain an in vitro assay system to correlate with in vivo tumorigenicity, many phenotypes have been reported for virus-transformed cells. However, Shields (1976) has concluded that generalizations about the nature of virus-transformed cells have not helped in understanding the processes of viral transformation and in vivo tumorigenicity. Gallimore et al. (1977) examined a number of Ad2-transformed rat cells and demonstrated a negative correlation with expression of a cell surface protein (LETS) and tumorigenicity in adult nude mice. However, there was also a negative correlation with the amount of viral T antigen expressed by the transformed cells and in vivo tumorigenicity.

It is clear from studies on DNA tumor viruses, mainly adenovirus, polyoma and SV40, that the viral specified early proteins (e.g. T antigens) play a specific role in cell transformation and possibly in vivo tumorigenicity of the virus. It has already been noted (section 1.8) that some early proteins are involved in both transformation and viral DNA replication in adenovirus infected cells. In addition, a limited number of studies with oncogenic DNA viruses have shown that these early

proteins play a role in the induction of host cell DNA replication by the viruses. Thus the initiation of transformation of host cells during abortive infection may involve stimulation of host cell DNA replication under conditions which restrict normal cell DNA replication.

The best studied effect by adenoviruses on host cell DNA replication is during human Ad12 infection of hamster cells (Strohl, 1973). The Ad12 infection is abortive because viral DNA and late proteins are not synthesized and there is no production of infectious virus. However, early viral mRNA and viral specific protein, including the Ad12 specific tumor antigen (T antigen), are synthesized in hamster cells (Strohl, 1973). Adenovirus type 12 also induces transformation of hamster cells. The transformed cells were selected by growth in soft agar and have a characteristic morphology of virus-transformed cells, a low serum requirement, synthesis of T antigen in 33/37 isolates and they can produce tumors in hamsters (Strohl, 1973), as can the parental, non-transformed cells.

Infection of serum-starved, G1-arrested hamster cells by Ad12 resulted in the stimulation of host cell DNA replication and a subsequent increase in mitotic activity (Strohl, 1969), a process which required an infectious virus inoculum (Raška et al., 1971) and expression of the Ad12 T antigen (Strohl, 1969). The host cell DNA stimulation was accompanied by a rise in the levels of some host DNA replication enzymes (Zimmerman et al., 1970). The newly replicated host DNA later appeared in metaphase chromosomes of mitotic cells (Strohl, 1969), most of which were highly disorganized. The replication of host DNA in a non S phase state, induced by adenovirus, probably explains the induction of chromosome aberrations in host cells induced by Ad12, which have been described by zur Hausen (1973).

Stimulation of host cell DNA replication by adenoviruses has also been demonstrated in human Ad5-infected mouse cells (H.B. Younghusband, C. Tyndall and A.J.D. Bellett, submitted for publication; B.W. Stillman and A.J.D. Bellett, Cold Spring Harbor Symp. Quant. Biol., 43: in press), in which early viral protein synthesis and viral DNA replication occur, with very little late viral protein synthesis and virus production. Adenovirus type 5 also induced chromosome aberrations in mouse cells (A.J.D. Bellett, personal communication). Pater and Mak (1975) have reported that Ad12 in human KB cells (permissive for Ad12) selectively synthesizes host repetitive DNA sequences and inhibits replication of bulk host cell DNA. The host cell DNA synthesis is generally inhibited by adenovirus infection of permissive cells (Piña and Green, 1969), however, Horwitz et al. (1973) have suggested that host cell DNA might be stimulated early in productive infection and prior to synthesis of viral DNA.

Genetic studies with polyoma and SV40 virus have indicated that at least two early proteins (large T and small t antigens) are involved in determining the transformed state (Eckhart, 1977; Fluck et al., 1977; Bouck et al., 1978; Feunteun et al., 1978; Sleight et al., 1978; Schlegel and Benjamin, 1978). One of these, the A gene product, or T antigen, has a direct role in viral DNA replication and stimulation of host cell DNA replication, and may be involved in regulation of cell DNA replication and transcription (see Tegtmeyer and Rundell, 1977, for a review). Schlegel and Benjamin (1978) have shown that polyoma host range mutants defective in t antigen (hr-t), have a mitogenic activity that could stimulate host cell DNA replication in rat cells. This mitogenic activity is probably due to the T antigen. However, the hr-t mutants have lost the ability to induce morphological transformation (Schlegel and Benjamin, 1978).



Studies using temperature sensitive mutants of SV40 and polyoma virus defective in T antigen (see Tegtmeyer and Rundell, 1977) have shown that the T antigen can regulate the transformed phenotype, but this depends upon the method of selection of the transformed cells (Seif and Cuzin, 1977; Rassoulzadegan et al., 1978). When SV40 ts-A (defective in T antigen) transformants, that had been isolated as foci on cell monolayers, were incubated at the non-permissive temperature, the transformed phenotype reverted to a normal phenotype (Brugge and Butel, 1975; Kimura and Itagaki, 1975; Martin and Chou, 1975; Osborn and Weber, 1975; Tegtmeyer, 1975; Brockman, 1978). This effect correlates with the temperature sensitive regulation of cell DNA synthesis observed in SV40 ts-A transformants that had been selected as foci, which was observed by Butel and Soule (1978). In contrast, ts-A transformants selected as colonies growing in soft agar are temperature independent for expression of the transformed phenotype (Rassoulzadegan et al., 1978). The effect of temperature on cell DNA replication in these agar-selected transformants was not determined, however other SV40 ts-A transformed cell lines that had been selected in agar did not show temperature sensitive regulation of cell DNA replication (Robinson and Lehman, 1978). Although the determination of the transformed state is very complicated, it is clear that the T antigen has a function in initiation and maintenance of transformation in transformed cells selected as foci in liquid medium. T antigen has some role, possibly in initiation but not maintenance, of the transformation process in agar-selected cells. It is likely that stimulation of cell DNA replication by T antigen has a role in initiating the transformation process in both agar selected and focus selected cells. Then the effect of T antigen on the maintenance of transformation depends upon the method of selecting the cells.

Consistent with the idea that T antigen plays a role in determining control of host cell DNA replication and thus the initiation of the cell transformation process, in vitro studies have shown that purified SV40 T antigen binds to DNA and stimulates host cell DNA replication (Tjian et al., 1978; Tjian, 1978). When purified T antigen related protein was microinjected into cells, host cell DNA replication was observed by autoradiography (Tjian et al., 1978). This effect was also observed when SV40 early mRNA was microinjected into cells (Graessmann and Graessmann, 1976). The T antigen binds to double-stranded DNA (Carroll et al., 1974) including SV40 DNA (Reed et al., 1975; Jessel et al., 1976) and host cell chromosomes (D'Alisa and Gershey, 1978). Tjian (1978) has demonstrated that a T antigen related protein binds specifically to the origin of SV40 DNA replication.

Adenovirus T antigen may initiate the transformation process by inducing abnormal host cell DNA replication and possibly other changes. Continuous synthesis of T antigen may not be necessary to maintain the oncogenic state, since some tumorigenic hamster cell lines, which were derived from avian adenovirus-induced hepatomas, contain integrated adenovirus DNA sequences, but do not express T antigen (Stenback et al., 1973; May et al., 1978). However, other cell lines contained integrated viral sequences and expressed T antigen. Other avian adenovirus-induced hamster tumors, especially those at the site of injection such as fibrosarcomas, were shown to be T antigen negative (McCormick et al., 1971) since the sera from hamsters bearing primary (at the site of injection) or transplanted fibrosarcomas did not contain detectable antibody to T antigen by indirect immunofluorescence tests or complement fixation tests. However, these virus transformed hamster cells were not selected by the same procedure as that used by McAllister et al. (1969),

which is the usual selection used for adenovirus induced transformed cell lines (see above).

Adenovirus-specific tumor antigens have been isolated from both infected and transformed cells but have not been as well characterized as papovavirus T antigens. Gilead et al. (1976b) have identified 58,000 and 15,000 molecular weight proteins from Ad2-infected human KB cells (permissive) by specific immunoprecipitation with antisera against Ad2-transformed cells and a 58,000 molecular weight protein has been identified in a similar way from Ad5-infected human cells (Levinson and Levine, 1977a). This protein was different from other 'early' Ad5 proteins by peptide analysis (Levinson and Levine, 1977b). Adenovirus type 12 specific T antigens have been isolated from Ad12-transformed cells and have molecular weights of 80,000 (Biron and Raska, 1977) and 60,000 (Ledinko, 1978). The difference in the molecular weight is probably due to the different methods of measurement (sephadex chromatography and SDS-polyacrylamide gel electrophoresis respectively) and the 80,000 molecular weight protein was contaminated with other polypeptides which could alter the molecular weight estimation by chromatography. The 60,000 dalton protein found in rat cells transformed by a temperature-sensitive mutant of Ad12 (H12ts401) was only detected by immunoprecipitation at the permissive temperature and not at the non-permissive temperature. These cells also showed a temperature-sensitive transformed phenotype (Ledinko, 1978).

Studies on the proteins produced early in adenovirus infection, which have functions in adenovirus DNA replication and cell transformation, or both, should enable a better understanding of how adenoviruses are able to alter cellular growth properties in vitro and induce tumors in vivo.



2.1 Virus and cells

Human adenovirus type 5 (HAd5) was grown in monkey kidney cells. Biochemical experiments, including plaque labeling experiments, were done in monkey kidney cells. Cells were grown in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FCS). Virus titrations were done either by the fluorescent antibody technique (Phillips, 1961; see Chapter 3.2c) or by plaque assays on monkey kidney cells. Both these assays gave similar virus titers.

Monkey kidney cells (MDCK) were used as a target cell for HAd5. Cells were grown in MEM supplemented with 10% FCS. After 3 to 5 days' incubation, the cells were harvested and the virus was purified by density gradient centrifugation.

CHAPTER 2

2.2 Purification of virus

MATERIALS AND METHODS

Monolayers of monkey kidney cells were grown in MEM supplemented with 10% FCS. Cells were harvested and the virus was purified by density gradient centrifugation. The virus was then purified by sucrose density gradient centrifugation. The purified virus was then used for the experiments described in this chapter.

## 2.1 Viruses and cells

Human adenovirus type 5 (Ad5) was grown in monolayers of human KB cells. Biochemical experiments, including pulse-labelling experiments, were done in monolayers of human HEK cells. Cells were grown in modified Eagle's medium ('Autopow', Flow Laboratories) supplemented with 10% calf serum. Virus titrations were done either by the fluorescent antibody technique (Philipson, 1961; see Chapter 5.2c) or by plaque assays on monolayers of HEK cells using the method of Williams (1970). Both these assays gave similar titres of virus stocks.

Chick embryo lethal orphan virus (CELO virus), an avian adenovirus, was grown in 9 to 10 day embryonated eggs. After 2 to 3 days' incubation, the allantoic fluid was harvested. Virus was purified by the method described by Younghusband and Bellett (1971).

## 2.2 Purification of virus

Monolayers of human KB cells were infected with 1 to 5 plaque-forming units (p.f.u.) of Ad5 in modified Eagle's medium without calf serum and absorbed to the cells for 1 hr at 37°C. Extra medium without calf serum was then added and the cells were incubated at 37°C for 2 to 4 days, until all cells had rounded up and came off the glass. When labeled virus was prepared, the medium was replaced 8 hr post infection (p.i.) with either phosphate-free medium containing  $^{32}\text{P}$ , or normal medium without calf serum containing  $^3\text{H}$ -thymidine. Variable amounts of radioactive label were used. Cells were collected, washed with phosphate-buffered saline (PBS), and frozen and thawed three times in 10mM Tris, pH 7.2. The lysed cells were centrifuged at 2000 rpm for 15 minutes on a bench centrifuge and virus was purified from the supernatant fluid.

The supernatant was layered onto a preformed gradient of CsCl (equal volumes of CsCl, density 1.45 g/ml and density 1.33 g/ml in 10mM Tris, pH 7.2) and centrifuged at 20,000 to 30,000 rpm in either the SW27, SW41 or SW50.1 Spinco rotors for 2 hr at 4°C. The visible virus band was collected and recentrifuged twice in similar gradients (average density of 1.35 g/ml) in the Spinco SW50.1 rotor, at least one of which was centrifuged overnight at 30,000 rpm at 4°C. The purified virus was dialysed against 1xSSC (0.15M NaCl, 0.015M Na Citrate) or STE (0.1M NaCl, 0.05M Tris, pH 7.2, 0.001M EDTA) and stored at 4°C.

### 2.3 Purification of viral DNA

Protease VI (1 mg/ml) was added to the virus preparation and incubated at 37°C for  $\frac{1}{2}$  hr, then sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5% (g/100 ml) at 37°C for a further hour. The mixture was extracted 3 times with phenol, buffered with 0.1M NaCl, 0.001M EDTA and 0.01M Tris, pH 8. The DNA in the aqueous phase was dialysed against STE.

### 2.4 Purification of bacteriophage T7 DNA

Bacteriophage T7 was grown on *Escherichia coli* strain C using M9 medium (Kornberg et al., 1959), supplemented with 20 µg/ml thymidine, reduced to 0.5 µg/ml when  $^3\text{H}$ -thymidine was added. DNA was prepared from purified virions by the method of Ritchie et al. (1967).

### 2.5 Purification of DNA-protein complex from virions

Ad5-infected HEK cells were labeled with  $^{32}\text{PO}_4$  for 2 days and virus purified as described previously (Younghusband and Bellett, 1971, see above). DNA-protein complex was purified by equilibrium CsCl centrifugation



in the presence of 4M guanidine hydrochloride following the method of Rekosh et al. (1977) and phenol extraction. Purified virus was mixed with 4.076g CsCl, 3.016g guanidine hydrochloride, 0.2% sarkosyl (final concentration) and STE (0.1M NaCl, 0.001M EDTA and 0.05M Tris, pH 7.2) so that 8 ml weighed 11.816g. Centrifugation was for 64 hr at 33,000 rpm in a Spinco Ti 50 rotor at 10°C. DNA-protein complex, which formed a sharp band in the middle of the gradient, was collected and dialysed against STE for at least 2 hr and extracted twice with redistilled phenol buffered with 0.1M NaCl, 0.001M EDTA and 0.1M Tris, pH 8. The purified DNA-protein complex was then dialysed extensively against STE to remove phenol. Similar results were obtained when DNA-protein complex was purified by Sepharose 2B column chromatography followed by CsCl/guanidine hydrochloride/sarkosyl equilibrium centrifugation.

## 2.6 Chromatography on benzoylated, naphthoylated DEAE-cellulose

BND-cellulose was rehydrated with 0.3M NaCl in TE buffer (0.01M Tris, pH 8 and 0.01M EDTA) containing 20% ethanol, centrifuged and washed once by re-centrifugation in 0.3M NaCl in TE buffer. A 1 ml column in a 2.5 ml syringe was washed extensively with 0.3M NaCl in TE buffer until the optical density at 260 nm of the effluent was less than 0.05. DNA was loaded onto the column in TE buffer containing 0.3M NaCl; addition of SDS to the loading buffer prevents binding of the DNA-protein complex to the column. The column was then washed with 1 ml of TE buffer containing 0.3M NaCl and 0.1% sodium dodecyl sulphate (SDS); 0.1% SDS does not reverse the binding of complex at this stage, but it increases the recovery of protein-free DNA from the column. Double-stranded, protein-free DNA was eluted with TE buffer containing 1M NaCl and 0.1% SDS and single-stranded and partially single-stranded DNA was eluted with TE buffer containing 1M NaCl, 0.1% SDS and 2% caffeine (Bellett and Younghusband, 1972; van der Eb,

1973). Single-stranded DNA was prepared by addition of NaOH (0.15M final concentration) to DNA that was made at least 10mM in EDTA. After 5 minutes at room temperature, the DNA was diluted 10-fold at 0°C with 0.3M NaCl in TE buffer and when necessary brought to approximately pH 8 with acetic acid. The DNA was then chromatographed as described above. Unless otherwise stated, recovery of DNA from the columns was, or close to, 100%.

DNA-protein complex was eluted by either protease VI (1 mg/ml) treatment on the column (30 minutes at 37°C) followed by the above procedure for elution, or by elution with TE buffer containing 8M urea and 1% SDS. When required, DNA was mixed with 2 volumes of ethanol, stored at -20°C overnight or in dry ice-ethanol for 10 minutes, and pelleted by centrifugation. The DNA pellet was resuspended in TE buffer containing 0.3M NaCl, and 1 mg/ml protease VI when required.

## 2.7 Restriction enzyme digestions and agarose gel electrophoresis

Digestions with restriction endonucleases EcoRI and HpaI were done at 37°C for 1 hr in a buffer containing 0.01M Tris, pH 7.5, 0.01M MgCl<sub>2</sub> and 0.01M 2-mercaptoethanol. Digestion with restriction endonuclease HindIII was done under similar conditions in a buffer containing 0.05M NaCl, 0.006M MgCl<sub>2</sub> and 0.006M Tris, pH 7.4. The reaction was stopped by the addition of EDTA, pH 7.2 to a final concentration of 20mM. Samples for agarose gel electrophoresis were made up to 10% glycerol and 0.05% bromophenol blue and subjected to electrophoresis through either a 1% agarose horizontal slab gel (15cm x 15cm x 0.6cm) at 20V for 20 hr or a 1% agarose tube gel (0.5cm x 15cm) at 50V for 7 hr at room temperature. The electrophoresis buffer contained 0.04M Tris, pH 8.2, 0.005M sodium acetate and 0.01M EDTA. Gels were either stained with 0.05 µg/ml ethidium bromide in electrophoresis buffer and photographed under UV light,

or dried down for autoradiography using Kodak RP X-Omat X-ray film at room temperature.

## 2.8 Determination of radioactivity

Acid-insoluble radioactivity was determined by spotting 50 $\mu$ l of a sample onto a Whatman 3mm filter, washing the filter 3 times in 10% trichloroacetic acid at 0°C, and then twice with ethanol. After drying the filter, it was placed in 5 ml of toluene based scintillation fluid (toluene containing 5g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per litre) and radioactivity determined in a Packard series 3000 liquid scintillation counter. Acid-soluble radioactivity (after exonuclease III digestion) was determined by the method described by Younghusband and Bellett (1971). A 0.1 ml sample was mixed with 0.4 ml of calf thymus DNA (1 mg/ml) and 0.5 ml of 10% trichloroacetic acid at 0°C. After 15 minutes in ice, the samples were centrifuged for 30 minutes at 17,000 x g at 0°C. A 0.5 ml amount of supernatant was mixed with 1 ml of water and 10 ml of Triton X scintillation fluid (62.5% toluene, 37.5% triton X-100, containing 5 g of 2,5-diphenyloxazole per litre and 0.3g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per litre).

When  $^3\text{H}$  and  $^{32}\text{P}$  were counted in the same sample, standards of each isotope in the same batch of scintillation fluid were also counted. Counts were converted to counts per minute and corrected for background and  $^{32}\text{P}$  contamination of the  $^3\text{H}$  channel (usually about 2.5%) by the use of a PDP 8/1 computer.



## 2.9 Methods described in other chapters

Some experimental procedures, which were used for specific experiments in following chapters, are indicated below:

- . Purification of  $^3\text{H}$ -thymidine labeled Ad5 DNA. (i)  $^3\text{H}$ -thymidine pulse-labeled DNA (method of Hirt, 1967); (ii)  $^{32}\text{P}/^3\text{H}$  labeled LH DNA, and (iii) alkaline sedimentation of pulse-labeled DNA (Chapter 3)
- . Purification of  $^3\text{H}$ -thymidine pulse-labeled Ad5 DNA-protein complex (Chapter 5)
- . Purification of  $^3\text{H}$ -thymidine labeled cell DNA (Chapter 5)
- . Chromatography on hydroxylapatite (Chapter 3)
- . Exonuclease III digestion and annealing of DNA (Chapter 3)
- . Electron microscopy of DNA (Chapter 3)
- . Staining of infected cells with fluorescent antibody (Chapter 5)

## 2.10 Enzymes

Restriction endonuclease EcoRI was prepared by Dr A.J.D. Bellett, using the method of Yoshimori (1971), from *Escherichia coli* strain RY-13. Some batches of restriction endonuclease HpaI were a gift from Dr K.D. Brown (University of Sydney, Sydney, NSW) and others were prepared from *Haemophilus parainfluenzae* using the method described by Sharp et al. (1973). Restriction endonuclease HindIII was prepared from *Haemophilus influenzae* strain D by ammonium sulphate precipitation of disrupted cell extracts, followed by chromatography in sephacryl S-200 (Pharmacia Fine Chemicals) and DEAE-cellulose column chromatography (Dr R. Appels, CSIRO Division of Plant Industry, Canberra, personal communication) in collaboration with Dr R. Appels.

Exonuclease III from *E.coli* was a gift from Dr I.R. Lehman, Stanford University, California.

Protease type VI was purchased from Sigma Chemical Co. A stock solution (10 mg/ml in H<sub>2</sub>O) was incubated at 56°C for 2 hr prior to use and stored at -18°C. Ribonuclease A was purchased from Worthington Biochemical Corporation. A stock solution (2 mg/ml in 0.05M sodium acetate, pH 5) was heated to 100°C for 19 minutes and stored at -18°C.

## 2.11 Reagents

Guanidinium hydrochloride (GuHCl) was recrystallized by slowly cooling a boiling solution of ethanol saturated with guanidinium hydrochloride (BDH Biochemicals). The saturated ethanol solution was filtered through Whatman No. 1 filter paper and put at -20°C. The crystals were filtered and washed with ethanol and then air dried.

Phenol was redistilled in an atmosphere of N<sub>2</sub> and stored under N<sub>2</sub> in sealed tubes at -18°C.

Benzoylated, naphthoylated DEAE-cellulose (Serva, Heidelberg, Germany) was rehydrated and columns prepared as described above.

Cyclohexamide and electrophoresis grade agarose were purchased from Sigma Chemical Co.; 5-bromodeoxyuridine (BUdR) and 5-fluorodeoxyuridine were purchased from Calbiochem; sarkosyl (NL-97) was a gift from Ciba-geigy.

All other chemicals were analytical grade.

Radioactive compounds: Methyl-<sup>3</sup>H-thymidine was 40-90 Ci/mMole and was purchased from the Radiochemical Centre, Amersham, England. For high specific activity labeling, the <sup>3</sup>H-thymidine was dried down under vacuum before use. L-lysine-4,5-<sup>3</sup>H was 60 Ci/mMole and was purchased from the Radiochemical Centre, Amersham. <sup>32</sup>P (carrier-free) was purchased from the Australian Atomic Energy Commission, Sydney.

## R E S U L T S

### Evidence against self-primed initiation

of the 5' ends of replicating adenovirus



### 3.1 Introduction

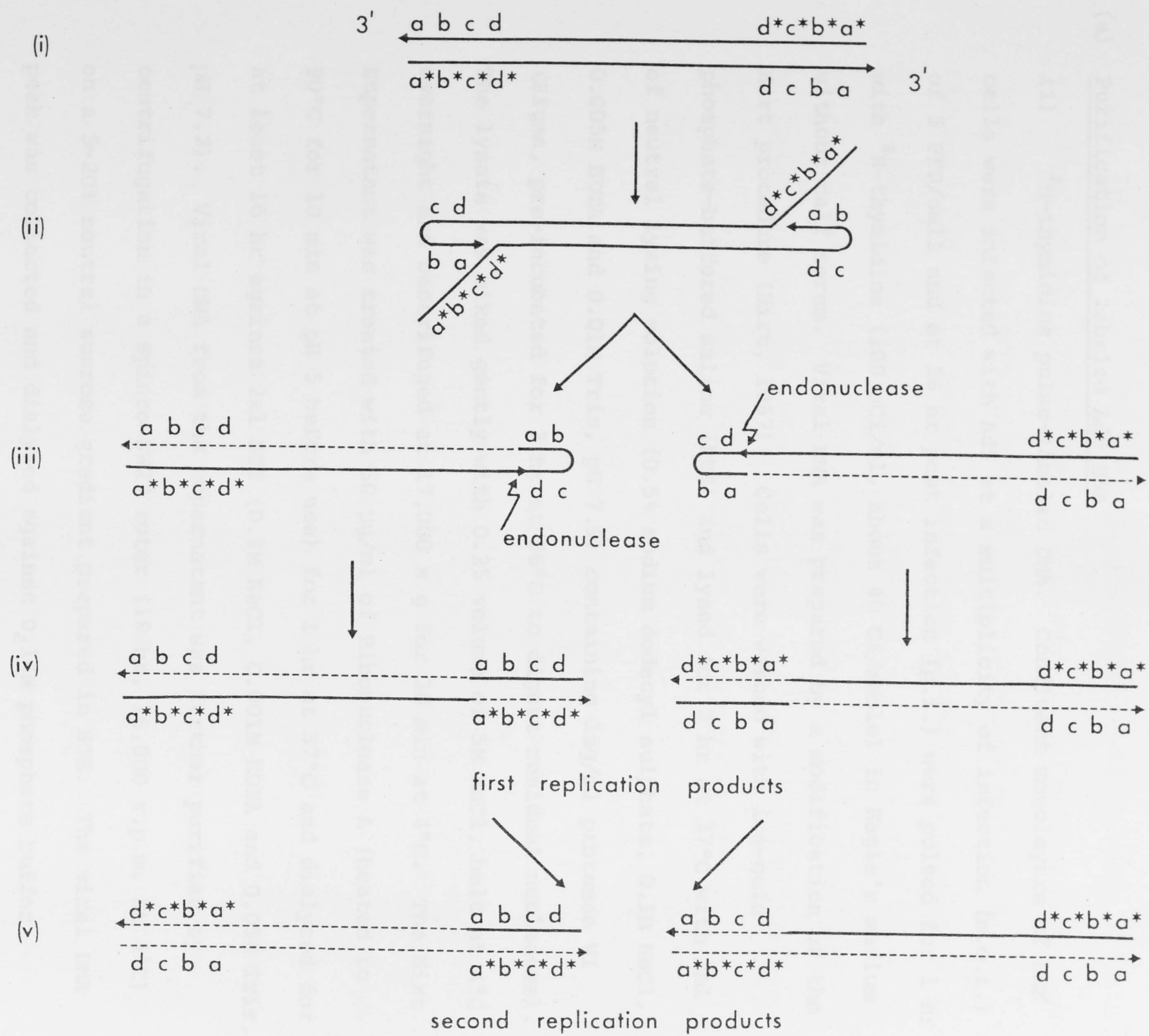
The mechanism of replication of the 5' ends of linear DNA chromosomes remains unclear (Bellett and Youngusband, 1972; Watson, 1972), as the known DNA polymerases, which act in the 5' to 3' direction, require a free 3'-OH as a primer for chain elongation. If a short ribonucleotide sequence primed DNA synthesis, its subsequent removal would leave no primer for gap fill synthesis at the 5' end. Most linear bacteriophage DNA molecules therefore have terminal repeats or single-stranded complementary ends, which allow formation of circular or concatemeric replication intermediates (Chapter 1). Several interesting models have been proposed to explain replication at the 5' ends of eukaryote chromosomes and linear DNA molecules which cannot circularize, and these have been discussed in detail in Chapter 1. There is now considerable evidence for a terminal self-complementary 'hairpin' loop which primes replication of the DNA of minute virus of mice (MVM) and adeno-associated virus (AAV) (see section 1.4) and hairpin priming may well be a general mechanism for replication of linear DNA molecules that are unable to circularize or form concatemers (Cavalier-Smith, 1974; Bateman, 1975; Tattersall and Ward, 1976).

Wu et al. (1977) and R.J. Roberts (personal communication to A.J.D. Bellett) have proposed a hairpin-primed model for replication of linear adenovirus DNA, which is based upon the model of Cavalier-Smith (1974) and is summarized in Figure 9. The hairpin-priming would occur at the ends of the molecule to initiate DNA replication, as the origins and termini are at or near the ends of the DNA (see section 1.6 for discussion). In this chapter, I will present evidence which is inconsistent with the hairpin-primed model for the initiation of adenovirus DNA replication.

Figure 9

Model for self-primed initiation of adenovirus DNA replication by a hairpin mechanism. (i) virion parental DNA (solid lines) showing inverted terminal repetition (sequence a b c); (ii) hairpin formation at the 3' end of either strand (sequence a complementary to sequence d); (iii) daughter strand synthesis in the 5' to 3' direction (broken lines) using the 3'-OH from the parental strand as primer, and site-specific endonuclease introducing a single-strand nick near sequence d; (iv) strand displacement within hairpin and completion of terminal 3' ends; (v) products of a second round of replication by the same mechanism.

Model proposed by Wu et al. (1977) and R.J. Roberts (personal communication to A.J.D. Bellett).





### 3.2 Methods

The following are methods used specifically in this chapter.

Other general methods were described in Chapter 2.

#### (a) Purification of labeled Ad5 DNA

(i)  $^3\text{H}$ -thymidine pulse-labeled DNA. Confluent monolayers of HEK cells were infected with Ad5 at a multiplicity of infection (m.o.i.) of 5 PFU/cell and at 16 hr post infection (p.i.) were pulsed for 1 hr with  $^3\text{H}$ -thymidine (100  $\mu\text{Ci/ml}$ , about 40 Ci/mMole) in Eagle's medium without calf serum. Viral DNA was prepared by a modification of the Hirt procedure (Hirt, 1967). Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed for 2 hr at  $37^\circ\text{C}$  with 2ml of neutral lysing solution (0.5% sodium dodecyl sulphate, 0.1M NaCl, 0.005M EDTA and 0.01M Tris, pH 7.9) containing 1mg/ml protease VI (Sigma, pre-incubated for 2 hr at  $56^\circ\text{C}$  to digest residual nucleases). The lysate was mixed gently with 0.25 volume of 5M NaCl, held at  $4^\circ\text{C}$  overnight and centrifuged at  $17,000 \times g$  for 30 min at  $4^\circ\text{C}$ . The Hirt supernatant was treated with 50  $\mu\text{g/ml}$  of Ribonuclease A (heated to  $90^\circ\text{C}$  for 10 min at pH 5 before use) for 1 hr at  $37^\circ\text{C}$  and dialysed for at least 16 hr against 2x1 STE (0.1M NaCl, 0.001M EDTA and 0.05M Tris, pH 7.2). Viral DNA from the supernatant was further purified by centrifugation in a Spinco SW27 rotor (18 hr, 16,000 r.p.m. at  $4^\circ\text{C}$ ) on a 5-20% neutral sucrose gradient prepared in STE. The viral DNA peak was collected and dialysed against 0.14M phosphate buffer pH 6.8 for hydroxylapatite chromatography.

(ii)  $^{32}\text{P}/^3\text{H}$ -labeled LH DNA. Confluent monolayers of HEK cells (approximately  $12 \times 10^6$  cells) infected with Ad5 at a m.o.i. of 5 PFU/cell and at 6 hr p.i. were labeled with  $^3\text{H}$ -thymidine (20  $\mu\text{Ci/ml}$ ,  $5 \times 10^{-7}\text{M}$  thymidine) in Eagle's medium without calf serum. At 16 hr

p.i., the cells were washed with phosphate-free Eagle's medium without calf serum to remove unincorporated  $^3\text{H}$ -thymidine, incubated in the same medium with  $^{32}\text{P}$  (200  $\mu\text{Ci/ml}$ ), 5  $\mu\text{g/ml}$  5-bromodeoxyuridine (BUdR,  $1.6 \times 10^{-5}\text{M}$ ) and 0.5  $\mu\text{g/ml}$  of 5-fluorouracil for 3 hr at  $37^\circ\text{C}$ , and viral DNA was prepared by the Hirt procedure. After ribonuclease treatment and dialysis against STE, the supernatant was mixed with STE and solid CsCl to give a final volume of 8ml and a density of 1.730g/ml, and centrifuged to equilibrium in a Spinco Ti 50 rotor at 33,000 rpm for 64 hr at  $20^\circ\text{C}$ . Approximately 20 fractions were collected and the light-heavy (LH) viral DNA peak pooled and dialysed against 0.14M phosphate buffer, pH 6.8 for hydroxylapatite chromatography.

(iii) Alkaline sedimentation of pulse-labeled DNA. Confluent monolayers of HEK cells in 50mm petri dishes were infected with 10 PFU/cell of Ad5, incubated for 18 hr in medium without serum, and then pulse-labeled with  $^3\text{H}$ -thymidine (120  $\mu\text{Ci/ml}$  for 15 min). The cells were washed twice with PBS at  $4^\circ\text{C}$ , scraped off the plastic, again washed in PBS and suspended in 0.2ml/dish of PBS. Each sample (0.4ml) was layered onto 0.3ml of alkaline lysis solution (0.3M NaOH, 0.5% SDS, 0.3M NaCl, 0.03M EDTA) on top of a 30ml alkaline sucrose gradient (5%-20% sucrose in 0.3M NaOH 0.7M NaCl). A further 0.4ml alkaline lysis solution was layered on top of the cell sample. After 1 hr at room temperature, the gradients were centrifuged for  $5\frac{1}{2}$  hr at 24,000 rpm and  $15^\circ\text{C}$  in the SW 25.1 rotor. Fractions (1ml) were collected from the top of the gradients by the use of an 'Auto-densiflow' (Nuclear-Chicago, Buchler Instruments Division). Acid-insoluble radioactivity was determined on a 20  $\mu\text{l}$  aliquot of each fraction.

(b) Chromatography on hydroxylapatite

(i) Purification of double-stranded DNA. DNA previously dialysed against 0.14M phosphate buffer, pH 6.8, was loaded onto a 1ml prewashed column in a 60°C water bath. The column was washed with approximately 10ml of 0.14M phosphate buffer and the DNA eluted with 0.4M phosphate buffer, pH 6.8. The peak of double-stranded DNA was collected and dialysed against 0.07M Tris, pH 8.0 and 0.001M MgCl<sub>2</sub> for exonuclease III digestion.

(ii) Analysis for self-complementary sequences. DNA dialysed against 0.05M phosphate buffer pH 6.8 was boiled for 10 min, chilled in ice, diluted to 5ml with 0.14M buffer and then chromatographed on a 2.5ml hydroxylapatite column as described above, except that the column was washed with 30ml 0.14M buffer before eluting partly double-stranded DNA with 0.4M buffer. Fractions (5ml or 10ml) were collected and radioactivity determined on duplicate 1ml aliquots of each.

(c) Exonuclease III digestion and annealing of DNA

The reaction conditions were those described by Rhoades et al. (1968). Samples from the limited digest were assayed for acid-soluble counts and annealed by procedures described previously (Younghusband and Bellett, 1971). *E.coli* exonuclease III was a gift from Dr I.R. Lehman, Department of Biochemistry, Stanford University Medical Center.

(d) Electron microscopy of DNA was done as described by Younghusband and Bellett (1971, 1972).



### 3.3 Results

#### (a) Test for terminal repetitions in newly replicated DNA predicted by the model

One consequence of the hairpin-primed model (Figure 9) is that with each round of replication, the terminal hairpin structure must be inverted, resulting in four different types of molecules differing in the order of the terminal sequences. As the adenovirus termini are not palindromic (Steenbergh et al., 1978; J.R. Arrand and R.J. Roberts, personal communication), the products of the first round of replication would have direct terminal repeats of two types (Figure 9, iv) and 50% of newly replicated intracellular DNA should therefore anneal to form circles after digestion of the 3' ends with exonuclease III in a similar way to linear molecules with a direct repeat, such as T7 DNA. I find that this is not the case.

Viral DNA was isolated from Ad5-infected HEK cells after labeling with  $^3\text{H}$ -thymidine from 16 hr to 17 hr post infection, and centrifuged on a 5%-20% neutral sucrose gradient (Figure 10). The peak of viral DNA indicated in Figure 10 was pooled and chromatographed on hydroxylapatite to concentrate the DNA and remove unincorporated  $^3\text{H}$ -thymidine, then subjected to limited digestion with the 3' specific exonuclease III. Samples from each limited digest were annealed extensively at a low concentration of DNA and run on 5%-20% neutral sucrose gradients, which should detect circular molecules as sedimenting faster than linear molecules. After digestion of 0.1%, 0.75%, 1.3% and 1.6% of the double-stranded molecules in one experiment, and 0.1%, 0.7%, 1.1% and 1.5% in a second experiment (Figure 11), circular molecules migrating faster than the linear Ad5 DNA were not observed. Samples from one experiment were also examined in the electron microscope, and no circular molecules were observed. In a control experiment, using similar digestion and annealing conditions, circle

Figure 10

Neutral sucrose gradient sedimentation of the Ad5 DNA labeled with  $^3\text{H}$ -thymidine (100  $\mu\text{Ci/ml}$ ) from 16 hr to 17 hr p.i. The Hirt supernatant (Hirt, 1967) from disrupted cells was layered onto a 5-20% neutral sucrose gradient prepared in STE and centrifuged in a Spinco SW27 rotor at 16,000 rpm for 18 hr at 4°C. Fractions (1 ml) were collected and acid-insoluble radioactivity determined on 20  $\mu\text{l}$  samples from each fraction. Fractions indicated by the bar were pooled and dialysed against 0.14M phosphate buffer for hydroxylapatite chromatography. Sedimentation was from left to right.

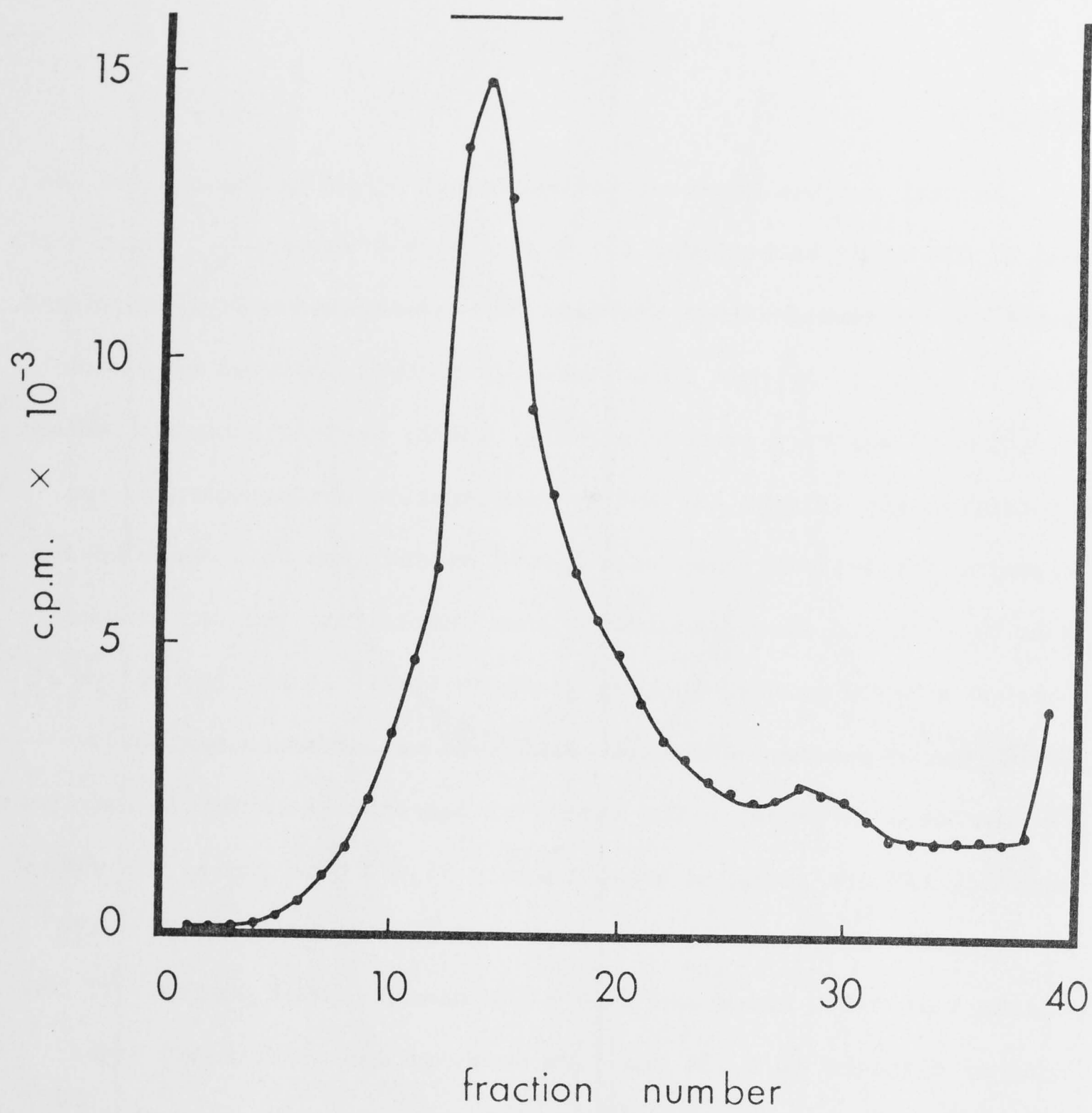
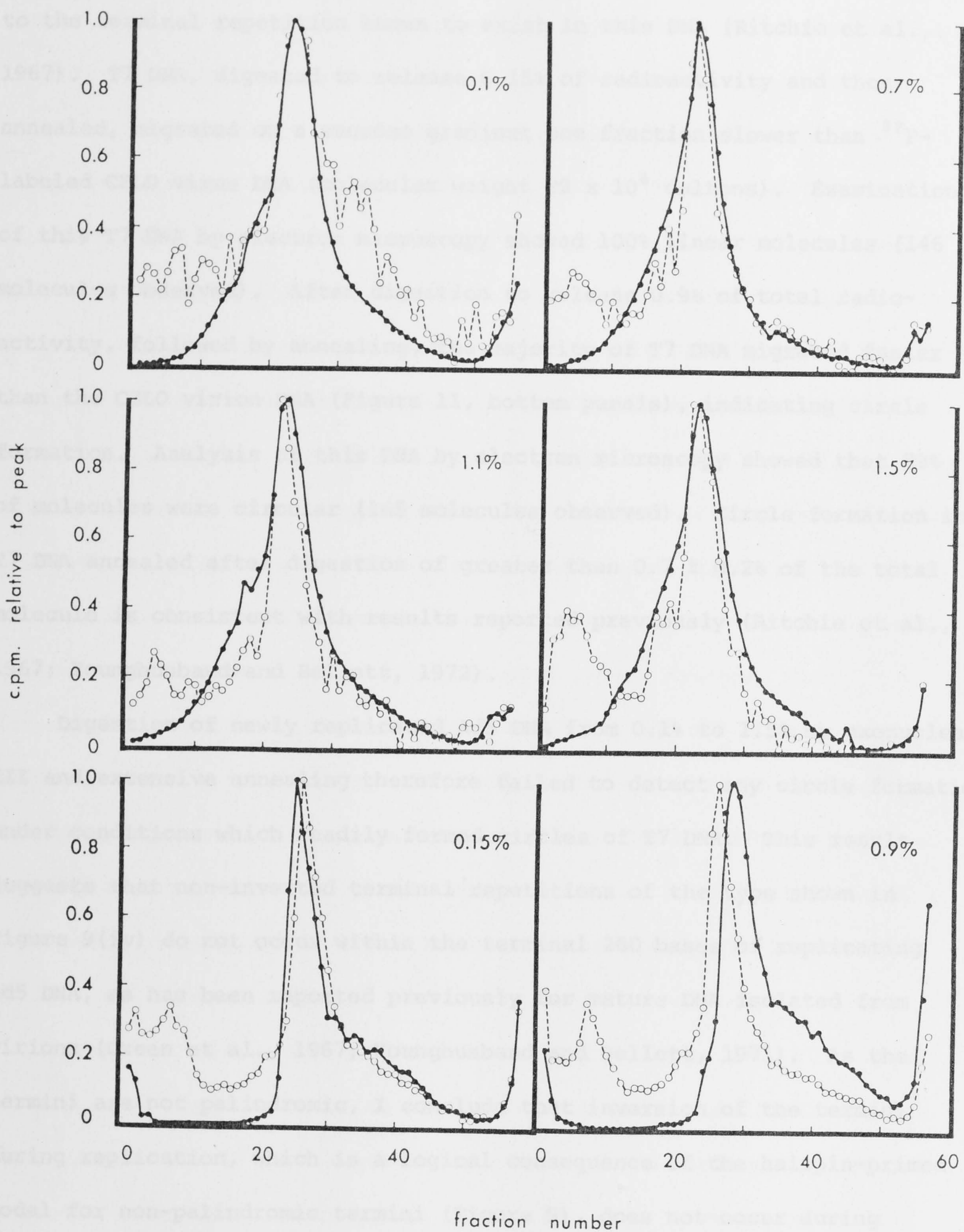




Figure 11

Neutral sucrose gradient sedimentation of pulse-labeled Ad5 DNA and T7 DNA after exonuclease III digestion and annealing. Human (HEK) cells were infected with 5 pfu/cell Ad5 and labeled with  $^3\text{H}$ -thymidine from 16 hr to 17 hr post infection. Viral DNA, isolated by a modification of the Hirt procedure (Hirt, 1967), neutral sucrose gradient centrifugation (Figure 10) and hydroxylapatite chromatography, was digested for various times with *E.coli* exonuclease III, annealed for  $\frac{1}{2}$  hr at  $65^\circ\text{C}$  and slowly cooled to room temperature and centrifuged through a 5-20% neutral sucrose gradient (SW27, 18 hr, 16,000 rpm at  $4^\circ\text{C}$ ). Top 4 panels:  $^{32}\text{P}$ -labeled Ad5 DNA was added as marker (o-----o);  $^3\text{H}$ -thymidine (●——●). The extent of digestion is shown in each panel; assuming Ad5 DNA contains approximately 35,000 base pairs, the extent of digestion from each 3' end would be 0.1% = 15 bases; 0.7% = 120 bases; 1.1% = 190 bases and 1.5% = 260 bases. Lower panels: T7 DNA control digested to 0.15% and 0.9% annealed and centrifuged under similar conditions (●——●), with  $^{32}\text{P}$  CELO virus DNA as marker (m.w.  $29 \times 10^6$ ). Sedimentation was left to right.



formation was observed in exonuclease III-treated T7 DNA (Figure 11), due to the terminal repetition known to exist in this DNA (Ritchie et al., 1967). T7 DNA, digested to release 0.15% of radioactivity and then annealed, migrated on a sucrose gradient one fraction slower than  $^{32}\text{P}$ -labeled CELO virus DNA (molecular weight  $29 \times 10^6$  daltons). Examination of this T7 DNA by electron microscopy showed 100% linear molecules (146 molecules observed). After digestion to release 0.9% of total radioactivity, followed by annealing, the majority of T7 DNA migrated faster than the CELO virion DNA (Figure 11, bottom panels), indicating circle formation. Analysis of this DNA by electron microscopy showed that 89% of molecules were circular (165 molecules observed). Circle formation in T7 DNA annealed after digestion of greater than  $0.7 \pm 0.2\%$  of the total molecule is consistent with results reported previously (Ritchie et al., 1967; Younghusband and Bellett, 1972).

Digestion of newly replicated Ad5 DNA from 0.1% to 1.5% by exonuclease III and extensive annealing therefore failed to detect any circle formation under conditions which readily formed circles of T7 DNA. This result suggests that non-inverted terminal repetitions of the type shown in Figure 9(iv) do not occur within the terminal 260 bases of replicating Ad5 DNA, as has been reported previously for mature DNA isolated from virions (Green et al., 1967; Younghusband and Bellett, 1971). As the termini are not palindromic, I conclude that inversion of the termini during replication, which is a logical consequence of the hairpin-primed model for non-palindromic termini (Figure 9), does not occur during adenovirus DNA replication.

(b) Test for covalent linkage of progeny to parental strand in replicating DNA

The self-primed model also requires covalent addition of progeny DNA to a hairpin on the parental strand (Figure 9). Some or all of pulse-



labeled viral DNA should therefore sediment faster than virion DNA in alkali, and should contain sequences that spontaneously renature on rapid transfer to conditions under which duplex DNA is stable. In fact, replicating adenovirus DNA, labeled under conditions where cell DNA replication is not detectable, sediments slower than virion DNA in alkali (Horwitz, 1971; Pearson and Hanawalt, 1971; Bellett and Younghusband, 1972). The intermediates expected if self-priming occurred would be from slightly longer than one genome to two genomes in length, their proportions depending on the relative rates of priming, chain elongation and endonuclease action. These forms would sediment at 36S to 46S in alkali (Studier, 1965). Some genome-length (35S) DNA is always present in this part of a gradient because of collection artefacts, diffusion and nonideal sedimentation, and could therefore mask a small amount of radioactivity in replication intermediates of the predicted type. However, such intermediates might still be detected as 36S to 46S DNA strands containing sequences able to renature spontaneously under appropriate conditions. Spontaneously renaturing sequences were tested for by chromatography on hydroxylapatite, which is able to detect a duplex section of 30 to 40 base pairs or longer in a DNA molecule that is partly single-stranded (Wilson and Thomas, 1973).

Confluent monolayers of HEK cells were inoculated with 10 PFU/cell of Ad5, incubated for 18 hr in medium without serum, and labeled with  $^3\text{H}$ -thymidine for 15 min (approximately half of the replication time of a molecule under these conditions). The cells were lysed on top of and centrifuged in two alkaline sucrose gradients, to one of which  $^{32}\text{P}$  Ad5 DNA was added. As reported previously (Horwitz, 1971; Bellett and Younghusband, 1972), the adenovirus replication intermediates sedimented more slowly than virion DNA (Figure 12). Fractions 11 to 16 of each gradient, representing DNA nominally sedimenting at 39S to 60S, contained

Figure 12

Alkaline sedimentation of pulse-labeled Ad5 DNA.

Viral DNA was labeled for 15 min at 18 hr p.i. and analysed on alkaline sucrose gradients.  $^{32}\text{P}$  Ad5 DNA ( $1.4 \times 10^5$  cpm) was added to gradient

(a). Sedimentation was from left to right. ●——●  $^3\text{H}$  replicating Ad5 DNA ( $5 \times 10^5$  cpm added to each gradient); ■-----■  $^{32}\text{P}$  Ad5 virion DNA.

Fractions 11 to 16 of each gradient (arrows, DNA nominally from 39S to 60S) were pooled, dialysed, and tested for spontaneously renaturing sequences (Table 1).

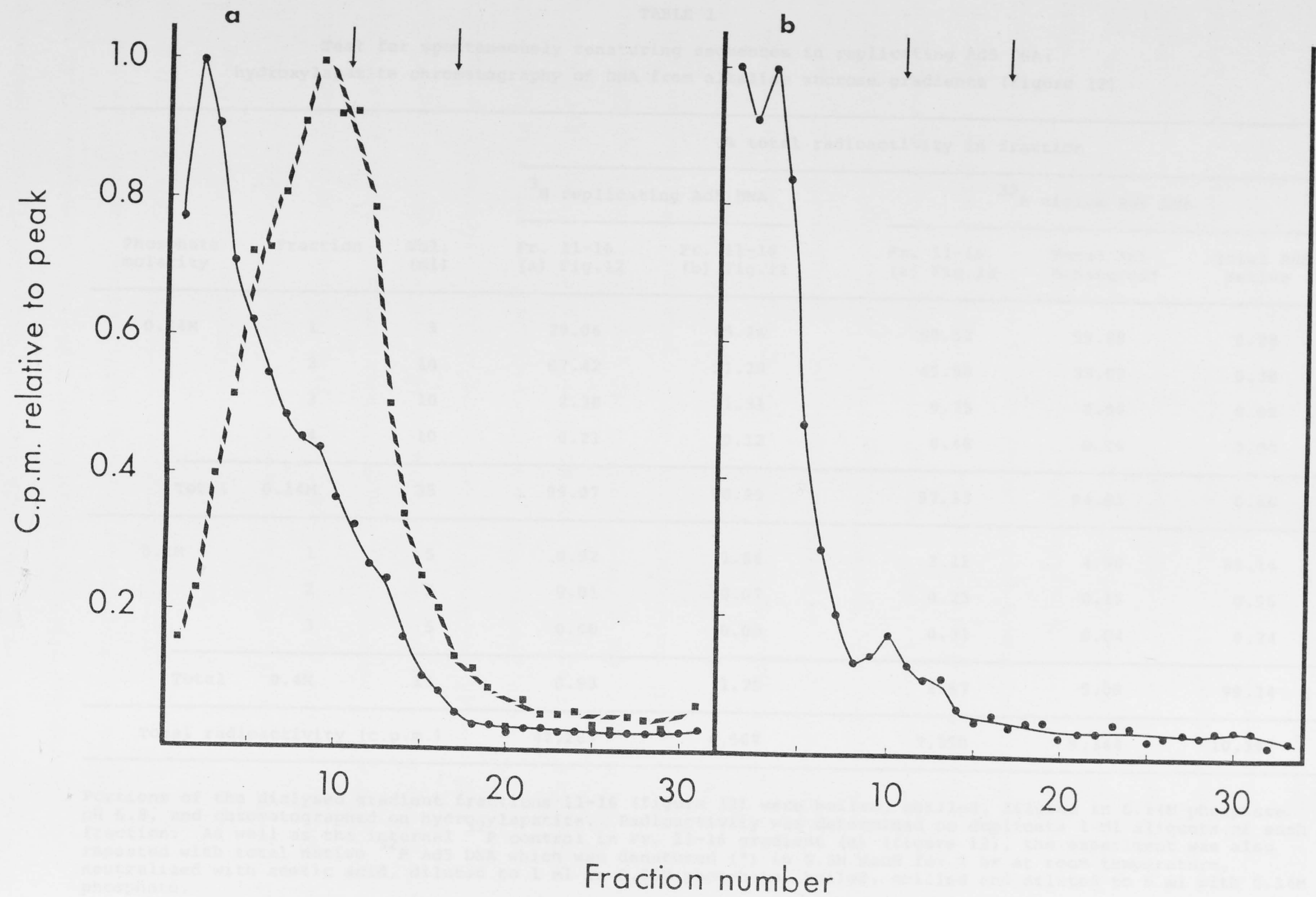




TABLE 1

Test for spontaneously renaturing sequences in replicating Ad5 DNA:  
hydroxylapatite chromatography of DNA from alkaline sucrose gradients (figure 12)

Phosphate molarity	Fraction	Vol. (ml)	% total radioactivity in fraction				
			<sup>3</sup> H replicating Ad5 DNA		<sup>32</sup> P virion Ad5 DNA		
			Fr. 11-16 (a) Fig.12	Fr. 11-16 (b) Fig.12	Fr. 11-16 (a) Fig.12	Total Ad5 Denatured*	Total Ad5 Native
0.14M	1	5	29.06	45.24	50.52	59.88	0.28
	2	10	67.42	51.28	45.58	35.03	0.38
	3	10	2.38	1.31	0.75	0.00	0.00
	4	10	0.21	0.12	0.48	0.00	0.00
Total	0.14M	35	99.07	98.25	97.33	94.91	0.66
0.4M	1	5	0.92	1.68	2.11	4.90	98.14
	2	5	0.01	0.07	0.25	0.15	0.96
	3	5	0.00	0.00	0.31	0.04	0.24
Total	0.4M	15	0.93	1.75	2.67	5.09	99.34
Total radioactivity (c.p.m.)			42,285	4,568	7,350	9,344	10,397

Portions of the dialysed gradient fractions 11-16 (figure 12) were boiled, chilled, diluted in 0.14M phosphate pH 6.8, and chromatographed on hydroxylapatite. Radioactivity was determined on duplicate 1 ml aliquots of each fraction. As well as the internal <sup>32</sup>P control in Fr. 11-16 gradient (a) (figure 12), the experiment was also repeated with total native <sup>32</sup>P Ad5 DNA which was denatured (\*) in 0.3M NaOH for 1 hr at room temperature, neutralized with acetic acid, diluted to 1 ml in 0.05M phosphate, boiled, chilled and diluted to 5 ml with 0.14M phosphate.

30.6% of  $^{32}\text{P}$  virion DNA (actually 35S), and 15.0% and 8.4% (average, 12%) of the two samples of replicating viral ( $^3\text{H}$ ) DNA. These fractions were pooled and dialysed against 0.05M phosphate pH 6.8 and samples were boiled, rapidly chilled in ice, diluted in 0.14M phosphate pH 6.8, and analysed for spontaneously renatured sequences by chromatography on hydroxylapatite. Only 1% to 2% (average 1.3%) of the  $^3\text{H}$ -replicating DNA was retained on hydroxylapatite in 0.14M phosphate, compared with approximately 3% of  $^{32}\text{P}$  Ad5 virion DNA from the same fractions of one of the alkaline sucrose gradients, or 5% of denatured virion DNA which had not been centrifuged (Table 1). More than 99% of native  $^{32}\text{P}$  Ad5 DNA was retained on hydroxylapatite in 0.14M phosphate. The gradient samples were also chromatographed on hydroxylapatite at 29°C with similar results. The fraction of replicating adenovirus DNA which had both the properties of the replicating intermediates predicted by the self-priming model (spontaneously renaturing DNA sedimenting at  $> 36\text{S}$  in alkali) was 0.16% (1.3% of 12%), which is less than the fraction of virion DNA (0.8%; 2.7% of 30.6%) which appears to have these properties because of experimental error. A replication intermediate with a duplex section (parental hairpin plus progeny DNA) of less than 30 to 40 base pairs would not have been detected in this experiment (Wilson and Thomas, 1973), but it seems unlikely that a shorter duplex could have the predicted function, and it is also inconsistent with the terminal sequence data (Steenbergh et al., 1978; J.R. Arrand and R.J. Roberts, personal communication).

(c) A test for a strand switch in newly replicated DNA

Another test for the self-priming model is based upon a prediction which is independent of the exact nature of the terminal sequences. It can be seen from Figure 9 that the model predicts a strand switch, in which the original parental 3' terminus becomes the 5' end of the progeny strand,

to be replaced by newly replicated DNA at the 3' end of the parental strand. Thus all newly replicated molecules should have newly replicated DNA at both 3' ends of the double-stranded DNA (Figure 9, iv). This was tested experimentally by prelabeling adenovirus type 5 (Ad5) DNA in HEK cells with  $^3\text{H}$ -thymidine (solid lines in Figure 9) and then transferring to medium containing  $^{32}\text{P}$  and 5-bromodeoxyuridine (BUdR) (dashed lines in Figure 9). If the model is correct, molecules that had undergone one round of replication in BUdR, isolated as a peak of hybrid (LH) density in CsCl, should have all their 3' ends labeled with  $^{32}\text{P}$ . However, 50% of the 3' ends of LH DNA should be labeled with  $^3\text{H}$  rather than  $^{32}\text{P}$  if there is no terminal strand switch during replication. The nature of the label at the 3' ends of LH DNA was examined by exonuclease III digestion.

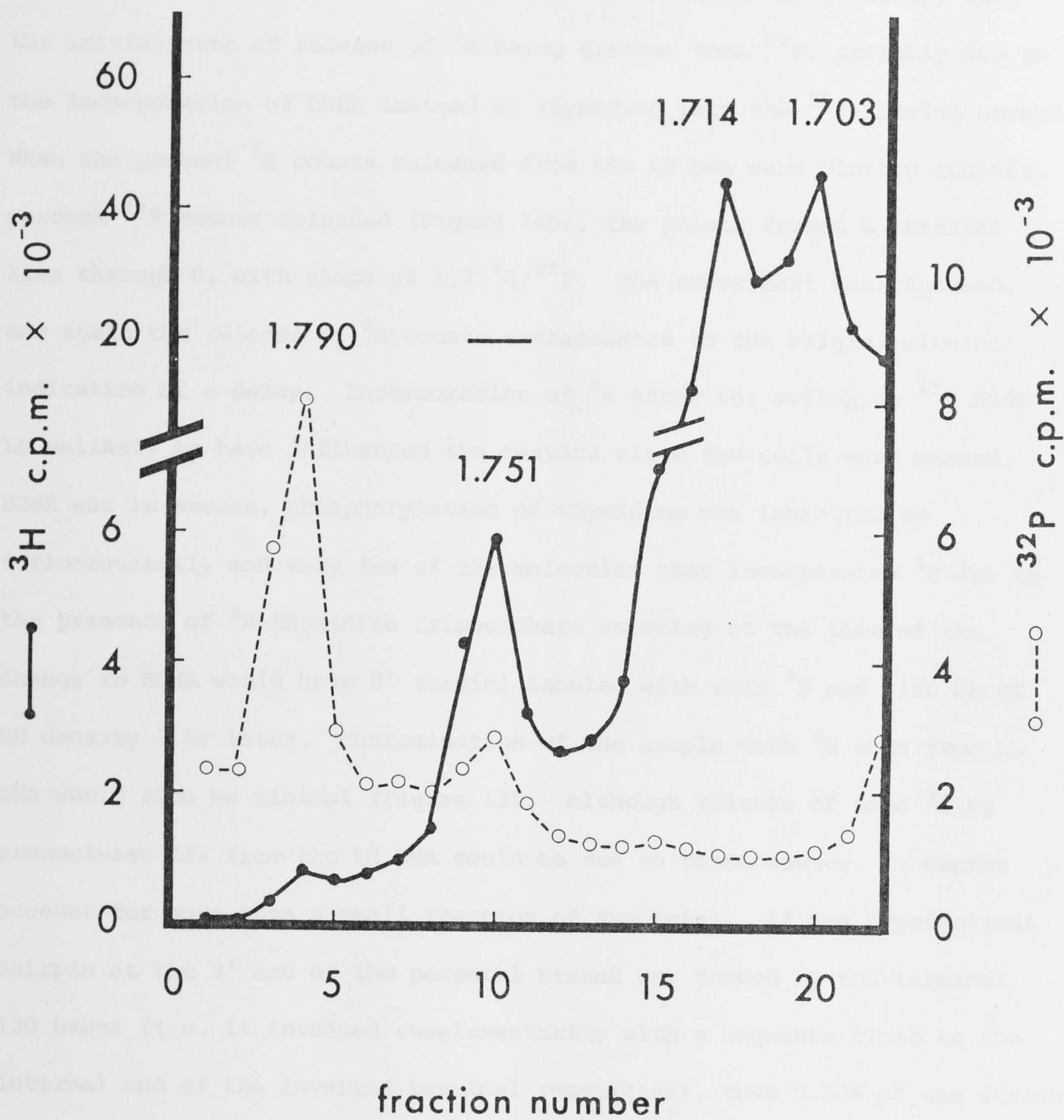
Isolation of  $^3\text{H}/^{32}\text{P}$  labeled LH DNA. HEK cells infected with Ad5 were labeled from 6-16 hr p.i. with  $^3\text{H}$ -thymidine to ensure that viral DNA was uniformly prelabeled. At 16 hr p.i., when cell DNA replication was not detectable, the replicating viral DNA was labeled for a further 3 hr with  $^{32}\text{P}$  and BUdR; the unincorporated  $^3\text{H}$ -thymidine was washed from the cell monolayer, then a 32-fold excess of BUdR over the original molar concentration of  $^3\text{H}$ -thymidine was added in the presence of 5-fluorouracil to inhibit phosphorylation of thymidine in vivo. Viral DNA was selectively extracted from the cells by the Hirt method and centrifuged to equilibrium in a neutral CsCl gradient with an initial density of 1.730g/ml (Figure 13). The viral LH DNA peak (1.751g/ml) was separated from viral HH (1.790g/ml) and LL (1.714g/ml) DNA and some remaining cell LL DNA (1.703g/ml). It was necessary to label viral DNA with  $^{32}\text{P}$  and BUdR for 3 hr at 37°C to obtain sufficient viral LH DNA. No peak of cellular LH DNA (density 1.740) was detectable. The viral LH DNA was centrifuged a second time in CsCl, purified further by chromatography on hydroxylapatite, and then analysed by partial digestion with exonuclease III.



Figure 13

CsCl equilibrium centrifugation of  $^{32}\text{P}/^3\text{H}$  labeled Ad5 DNA.

Ad5-infected HEK cells were labeled from 6-16 hr p.i. with  $^3\text{H}$ -thymidine (20  $\mu\text{Ci}/\text{ml}$ ) and then with  $^{32}\text{P}$  (200  $\mu\text{Ci}/\text{ml}$ ) and BUdR (5  $\mu\text{g}/\text{ml}$ ) in the presence of 5-fluorouracil (0.5  $\mu\text{g}/\text{ml}$ ) from 16-19 hr p.i. The Hirt supernatant from disrupted cells was centrifuged to equilibrium in CsCl in a Spinco Ti 50 rotor at 33,000 rpm for 64 hr at 20°C with initial density of 1.730 g/ml. The densities of the four main peaks were calculated from the refractive index: LL cell DNA,  $\rho = 1.703$  g/ml; LL viral DNA,  $\rho = 1.714$  g/ml; HH viral DNA,  $\rho = 1.790$  g/ml. The LH viral DNA peak,  $\rho = 1.751$  (indicated by bar) was collected, recentrifuged in another CsCl equilibrium gradient and dialysed against 0.14M phosphate buffer for chromatography on hydroxylapatite.



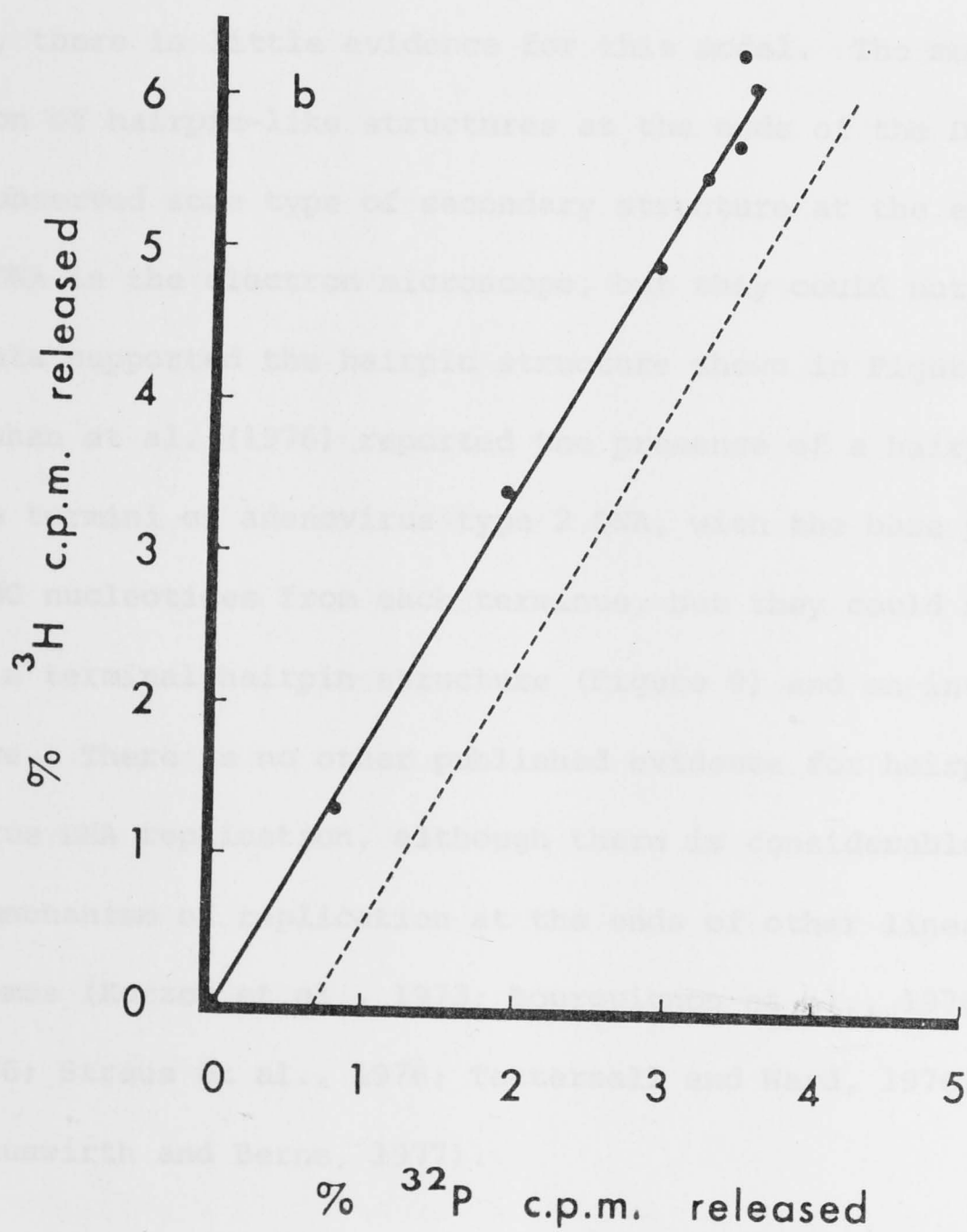
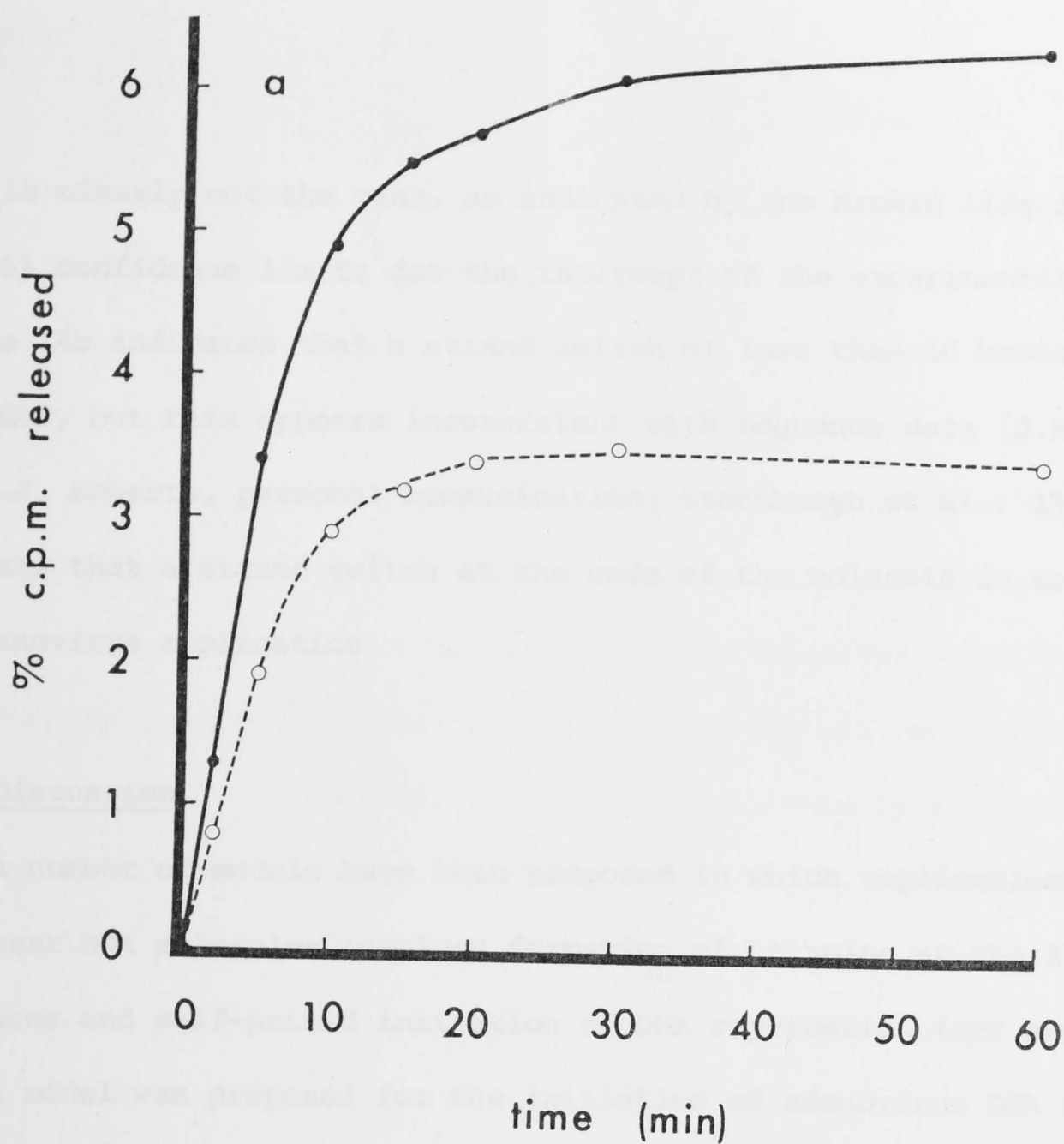
Characterization of the 3' end of viral LH DNA. The purified  $^3\text{H}/^{32}\text{P}$ -labeled LH viral DNA was subjected to digestion by *E.coli* exonuclease III. Figure 14a shows the release of acid-soluble counts with increasing digestion time. Both  $^3\text{H}$  and  $^{32}\text{P}$  counts were released immediately, with the initial rate of release of  $^3\text{H}$  being greater than  $^{32}\text{P}$ , probably due to the incorporation of BUdR instead of thymidine into the  $^{32}\text{P}$ -labeled strand. When the percent  $^3\text{H}$  counts released from the LH DNA were plotted against percent  $^{32}\text{P}$  counts released (Figure 14b), the points formed a straight line through 0, with slope of  $1.7 \text{ } ^3\text{H}/^{32}\text{P}$ . The experiment was repeated, and again the release of  $^3\text{H}$  counts extrapolated to the origin, with no indication of a delay. Incorporation of  $^3\text{H}$  after the switch to  $^{32}\text{P}$  BUdR is unlikely to have influenced the results since the cells were washed, BUdR was in excess, phosphorylation of thymidine was inhibited by 5-fluorouracil, and very few of the molecules that incorporated  $^3\text{H}$  due to the presence of  $^3\text{H}$ -thymidine triphosphate existing at the time of the change to BUdR would have 3' termini labeled with this  $^3\text{H}$  and also be of LH density 3 hr later. Contamination of the sample with  $^3\text{H}$  ends from LL DNA would also be minimal (Figure 13). Although release of some  $^3\text{H}$  by exonuclease III from the LH DNA could be due to these causes, it cannot account for more than a small fraction of the total. If the hypothetical hairpin at the 3' end of the parental strand was formed by the terminal 120 bases (i.e. it involved complementarity with a sequence close to the internal end of the inverted terminal repetition), then 0.34% of one strand, or 0.68% of the double-stranded molecule, should be released as  $^{32}\text{P}$  label before any  $^3\text{H}$  label was released, assuming Ad5 DNA contains approximately 35,000 base pairs. Thus, if the self-primed model is correct, the expected curve in Figure 14b would be a straight line with slope 1.7, passing through 0.68%  $^{32}\text{P}$  counts released and 0  $^3\text{H}$  counts released.



Figure 14

Digestion of  $^{32}\text{P}/^3\text{H}$  labeled LH Ad5 DNA by exonuclease III.

(a) Release, against time, of acid-soluble  $^3\text{H}$ -thymidine (●—●) from the light (L) strand and  $^{32}\text{P}$  (o-----o) from the heavy (H) strand from double labeled LH Ad5 DNA. (b) Data from (a) re-plotted as release of  $^3\text{H}$ -thymidine against release of  $^{32}\text{P}$  (●—●). The dashed line parallel to the observed curve and passing through (0.68, 0) is the expected curve if the strand switch in Figure 9 involves 120 base pairs, assuming Ad5 DNA contains approximately 35,000 base pairs.



This is clearly not the case, as indicated by the broken line in Figure 14b. The 95% confidence limits for the intercept of the experimental curve in Figure 14b indicates that a strand switch of less than 16 bases cannot be excluded, but this appears inconsistent with sequence data (J.R. Arrand and R.J. Roberts, personal communication; Steenbergh et al., 1978). This suggests that a strand switch at the ends of the molecule is not involved in adenovirus replication.

### 3.4 Discussion

A number of models have been proposed in which replication of the ends of linear DNA molecules involves formation of hairpins at the 3' terminal sequences and self-primed initiation of DNA replication (see section 1.4). Such a model was proposed for the initiation of adenovirus DNA replication (Wu et al., 1977; R.J. Roberts, personal communication to A.J.D. Bellett). However, there is little evidence for this model. The model requires formation of hairpin-like structures at the ends of the DNA. Wu et al. (1977) observed some type of secondary structure at the ends of adenovirus type 2 DNA in the electron microscope, but they could not conclude whether their data supported the hairpin structure shown in Figure 9. Similarly, Padmanabhan et al. (1976) reported the presence of a hairpin structure near the termini of adenovirus type 2 DNA, with the base paired region about 180 nucleotides from each terminus, but they could not distinguish between a terminal hairpin structure (Figure 9) and an internal hairpin structure. There is no other published evidence for hairpin priming in adenovirus DNA replication, although there is considerable evidence for such a mechanism of replication at the ends of other linear viral DNA chromosomes (Koczot et al., 1973; Bourguignon et al., 1976; Denhardt et al., 1976; Straus et al., 1976; Tattersall and Ward, 1976; Fife et al., 1977; Hauswirth and Berns, 1977).



Earlier work on adenovirus DNA replication is inconsistent with the self-primed model, which requires covalent addition of progeny DNA to the parental strands via a terminal hairpin (Figure 9). Pulse-labeled DNA should sediment faster than mature DNA in alkali and should contain sequences that spontaneously renature when returned to conditions in which the duplex is stable. However, replicating adenovirus DNA sediments more slowly than mature DNA in alkali (Horwitz, 1971; Pearson and Hanawalt, 1971; Bellett and Younghusband, 1972; Sussenbach and van der Vliet, 1972; Pettersson, 1973; van der Eb, 1973), and spontaneously renaturing intermediates have not been reported. There was no evidence of strands of replicating viral DNA more than genome length containing self-complementary sequences, when they were deliberately looked for (section 3.3b). It is possible that the hypothetical cross-linked intermediate is highly transient, and for this reason is not detectable. However, such intermediates were detected in replicating parvovirus DNA (Tattersall et al., 1973; Straus et al., 1976), and it seems simpler to conclude that they do not exist in replicating adenovirus DNA.

As another direct test of the self-primed model, I examined the 3' ends of newly replicated molecules of Ad5 DNA. According to the model (Figure 9) these should all consist of progeny material. However, about half the 3' ends of LH viral DNA molecules isolated after a shift from light ( $^3\text{H}$ ) to heavy ( $^{32}\text{P}$ ) medium consisted of parental ( $^3\text{H}$ ) DNA. The amount of  $^3\text{H}$  released by exonuclease III and the extrapolation of the curve to 0 time are not consistent with the self-primed model; one might expect a small release of  $^3\text{H}$  early in the reaction due to experimental errors of various kinds, but not complete agreement with the prediction for normal semiconservative replication without a strand switch.

For the self-primed model to produce identical sequences in every replication product, the terminal sequence not only has to form a hairpin, it also must be a palindrome (i.e. sequence ab complementary to sequence dc in Figure 9). However, the inverted terminal repetition is a unique, non-palindromic sequence in both Ad5 and Ad2 (Steenbergh et al., 1978; J.R. Arrand and R.J. Roberts, personal communication), and furthermore, it is unlikely that a hairpin sequence could exist within the terminal 190 base pairs of both ends of Ad5 DNA and have the predicted function in hairpin priming of DNA replication.

If the self-primed model applies at all to adenovirus DNA replication, it follows that the hairpin, which is assumed to prime DNA replication, must be only partially self-complementary and have a large single-stranded loop (i.e. sequence a complementary to sequence d, and sequence b not complementary to sequence c in Figure 9). Therefore, it can be seen from Figure 9 that only 25% of the replication products could be packaged in this form of the model, to produce unique virion DNA. In addition, half the molecules within the replicating pool should have direct terminal repetitions as shown in Figure 9 (iv). I was not able to detect terminal repetitions in replicating adenovirus DNA, although I could readily demonstrate them in bacteriophage T7 DNA. I conclude that the self-primed model shown in Figure 9 does not apply to the initiation of adenovirus DNA replication.

The work reported in this chapter has been published (Stillman et al., 1977, *Nature*, 269, 723) and since then Sussenbach and Kuijk (1978b) have published other evidence against the hairpin, self-primed model for adenovirus DNA replication. They pre-labeled Ad5 DNA with 5-bromodeoxyuridine to produce a pool of heavy-heavy viral DNA. Then the cells were pulse-labeled with <sup>3</sup>H-thymidine in the absence of BUdR and hybrid, light-heavy viral DNA molecules were isolated. Sussenbach and Kuijk (1978b)

could not find evidence for a strand switch when they examined the terminal restriction fragments for covalently linked  $^3\text{H}$ -thymidine and BUdR containing DNA, which was expected from a hairpin, self-primed model.

It is clear that a self-primed model does not apply to adenovirus DNA replication, even though the model is still considered by some as a possibility (Winnacker, 1978). Although the absence of hairpin-priming in adenovirus DNA replication does not indicate the mechanism by which the 5' ends of the progeny DNA are made, we have proposed an alternative model for adenovirus DNA replication in which the 55,000 molecular weight protein that is found on the termini of virion DNA is involved in initiation of DNA replication at the 5' end of nascent DNA strands (Rekosh et al., 1977; Chapter 6). In Chapter 5, I present evidence consistent with this model, using an assay for adenovirus DNA-protein complex which I describe in the next chapter.



CHAPTER 4

An assay for the adenovirus DNA-protein complex

#### 4.1 Introduction

Many proteins bind specifically, but non-covalently to either RNA or DNA and several methods have been used to detect these complexes. Jones and Berg (1966) and Riggs et al. (1970) used membrane filters to detect respectively, binding of RNA polymerase to bacteriophage T7 DNA and of the lactose repressor protein to lactose operon DNA. In both assays, high salt concentrations interfered with the binding of the complex to the filter. A number of examples of nucleic acid-protein complexes, in which the protein is covalently linked to the nucleic acid, were discussed in section 1.5. A filter binding assay for the adenovirus DNA-protein complex has been reported (Padmanabhan and Padmanabhan, 1977); however, this assay was not well characterized in that approximately 10% of the DNA-protein complex failed to bind to the filters and it was not reported if the complex, or DNA, could be recovered from the filters after binding. Furthermore, this assay could not distinguish between various forms of DNA in a DNA-protein complex, such as replicating or completed DNA molecules isolated during a short pulse with  $^3\text{H}$ -thymidine.

The adenovirus DNA-protein complex (Robinson et al., 1973; Robinson and Bellett, 1974) contains a 55,000 (55K) molecular weight protein covalently attached to the 5' ends of the virion DNA (Carusi, 1977; Rekosh et al., 1977). The complex was first detected when it was found that the protein caused non-covalent circularization of the DNA when viewed in the electron microscope (Robinson et al., 1973). Subsequently, the protein was shown to prevent DNA from entering agarose gels during electrophoresis (Brown et al., 1975; Sharp et al., 1976). However, both these assays for the adenovirus DNA-protein complex were not satisfactory for analysis of pulse-labeled viral DNA, mainly because they were not quantitative and also because detection of  $^3\text{H}$ -thymidine labeled replicating DNA was difficult. During the course of experiments designed to

determine whether intracellular replicating viral DNA had a protein attached to its termini, I found that virion DNA-protein complex bound tightly to a column of benzoylated, naphthoylated DEAE-cellulose (BND-cellulose). BND-cellulose chromatography had previously been used to separate completed and replicating pulse-labeled viral DNA. This chapter describes an assay for virion DNA-protein complex based on its binding to BND-cellulose. In the following chapter, I shall report experiments on replicating adenovirus DNA-protein complex using the BND-cellulose assay.

#### 4.2 BND-cellulose chromatography

This section describes the use of benzoylated, naphthoylated DEAE-cellulose chromatography as a technique for separating completely double-stranded DNA from partially single-stranded or completely single-stranded DNA. Kiger and Sinsheimer (1969) used BND-cellulose to separate replicating bacteriophage lambda DNA from completed phage DNA. The pulse-labeled phage DNA, in a buffer containing 0.3M NaCl, bound to BND-cellulose and was eluted by either gradient elution or step-wise elution. It was found that completely double-stranded DNA eluted in 1M NaCl and replicating DNA eluted in 1M NaCl plus 2% caffeine. Since single-stranded DNA also bound strongly to BND-cellulose and could be largely eluted by caffeine, Kiger and Sinsheimer (1969) concluded that the binding of replicating phage DNA was due to the single-stranded regions in this pool of DNA. A small amount of DNA remained bound to the column and could be removed by washing the column with 8M urea and 1% sodium dodecyl sulphate (SDS). Levine et al. (1970) used BND-cellulose chromatography to separate completed and replicating forms of SV40 DNA and also obtained some DNA that eluted with urea and SDS. Upon re-chromatography of this fraction of



the DNA, Levine et al. (1970) demonstrated that it eluted in 1M NaCl (one-third) and 2% caffeine (two-thirds), suggesting that this DNA bound non-specifically to the first column. Bellett and Younghusband (1972) and van der Eb (1973) have used BND-cellulose chromatography to separate completed and replicating adenovirus DNA molecules and an example of such a separation is given in this chapter.

During the course of my work using BND-cellulose chromatography, I have found it important to re-hydrate the BND-cellulose properly before use, and an advantage to use SDS in the elution buffers (see below and Chapter 2). By the use of these techniques, both completely double-stranded and replicating DNA could be quantitatively recovered from the columns with 1M NaCl or 1M NaCl plus caffeine buffers.

#### 4.3 Methods

Methods used in this chapter, including BND-cellulose chromatography, were given in Chapter 2.

#### 4.4 Results

##### (a) Recovery of DNA from BND-cellulose

As described above, Levine et al. (1970) and Bellett and Younghusband (1972) noted that while most double-stranded DNA could be eluted from BND-cellulose by 1M NaCl and most single-stranded DNA by 1M NaCl plus 2% caffeine, some DNA bound non-specifically and would only be eluted with 8M urea and 1% SDS. I have modified the conditions of BND-cellulose chromatography slightly and have found that double-stranded and single-stranded adenovirus DNA could be quantitatively recovered from BND-cellulose columns by elution with 1M NaCl plus SDS and by 1M NaCl plus 2% caffeine and SDS respectively. <sup>3</sup>H-thymidine labeled Ad5 DNA in 0.01M Tris, pH 8 and 0.01M EDTA was made 0.3M with NaCl and loaded onto a BND-

cellulose column. The column was washed with TE buffer containing successively 0.3M NaCl, 1M NaCl (with or without 0.1% SDS) and finally 1M NaCl plus 2% caffeine (with or without 0.1% SDS). It was found that double-stranded Ad5 DNA eluted in 1M NaCl and that the addition of 0.1% SDS to this buffer increased the recovery of DNA to 99% of the DNA added to the column (Table 2). Addition of 0.1% SDS to the caffeine and 1M NaCl did not increase the recovery of single-stranded Ad5 DNA that was denatured in 0.15 N NaOH and then neutralized by adding 3M acetic acid at 0°C.

The recovery of single-stranded Ad5 DNA could be increased to 93% (and greater with pulse-labeled Ad5 DNA, see Chapter 5) if the DNA was first made 10mM with EDTA, denatured with 0.15 N NaOH and then neutralized by diluting the DNA 1 in 20 with TE buffer containing 0.3M NaCl. This DNA was loaded onto a column at 4°C, washed with ice-cold 0.3M NaCl buffer and then DNA eluted from the column in 1M NaCl plus 0.1% SDS or 1M NaCl, 2% caffeine and 0.1% SDS. The reason for the different recovery rates obtained after denaturing DNA by two different methods is not known. DNA-protein complex isolated from Ad5 virions and assayed on BND-cellulose is described below.

(b) Isolation of virion DNA-protein complex

DNA-protein complex was prepared from purified adenovirus type 5 by disrupting the virus in the presence of 4M guanidine hydrochloride (GuHCl) and 0.2% sarkosyl, followed by equilibrium centrifugation in a CsCl density gradient containing 4M GuHCl and 0.2% sarkosyl, and then phenol extraction (Robinson and Bellett, 1974; Rekosh et al., 1977; see Chapter 2). Complex prepared in this way had the same properties as complex prepared by disrupting the purified virions in 4M GuHCl and removing virion protein on a Sepharose 2B column, followed by equilibrium

Table 2

Recovery of double-stranded and single-stranded Ad5 DNA  
from BND-cellulose columns

DNA	Recovery of Ad5 DNA (percent) <sup>(a)</sup>			
	1M NaCl		2% caffeine	
	- SDS	+ SDS	- SDS	+ SDS
<sup>3</sup> H-double-stranded <sup>(b)</sup>	64	99	-	-
<sup>32</sup> P-single-stranded <sup>(c)</sup>	0	0	64	66
<sup>32</sup> P-single-stranded <sup>(d)</sup>	-	1	-	92

(a) Recovery of DNA in different fractions from separate BND-cellulose columns in TE buffer containing 1M NaCl, + or - 0.1% SDS and 2% caffeine, 1M NaCl, + or - 0.1% SDS.

(b) <sup>3</sup>H-thymidine labeled, double-stranded Ad5 DNA (35,400 cpm) in 0.3M NaCl.

(c) <sup>32</sup>P-labeled single-stranded Ad5 DNA (31,400 cpm); <sup>32</sup>P-labeled Ad5 DNA in 0.1M NaCl, 0.05M Tris, pH 7.2 and 0.001M EDTA was denatured by addition of NaOH (0.15 N final concentration) for 5 minutes at room temperature and then neutralized at 0°C with 3M acetic acid. The columns were run at room temperature.

(d) <sup>32</sup>P-labeled single-stranded Ad5 DNA (10,600 cpm) was made 10mM with EDTA and denatured with NaOH (0.15 N final concentration) for 5 minutes at room temperature, then diluted 1 in 20 with ice-cold 0.3M NaCl and TE buffer. The DNA was loaded onto a BND cellulose column at 4°C, washed with 0.3M NaCl and then eluted with the indicated buffers at room temperature.

- indicates not done.



centrifugation of the DNA-protein complex in a CsCl/GuHCl/sarkosyl gradient (results not shown; method suggested by R.J. Roberts). Both techniques remove virion proteins not covalently bound to the DNA. The former technique was usually used for isolation of virion complex.

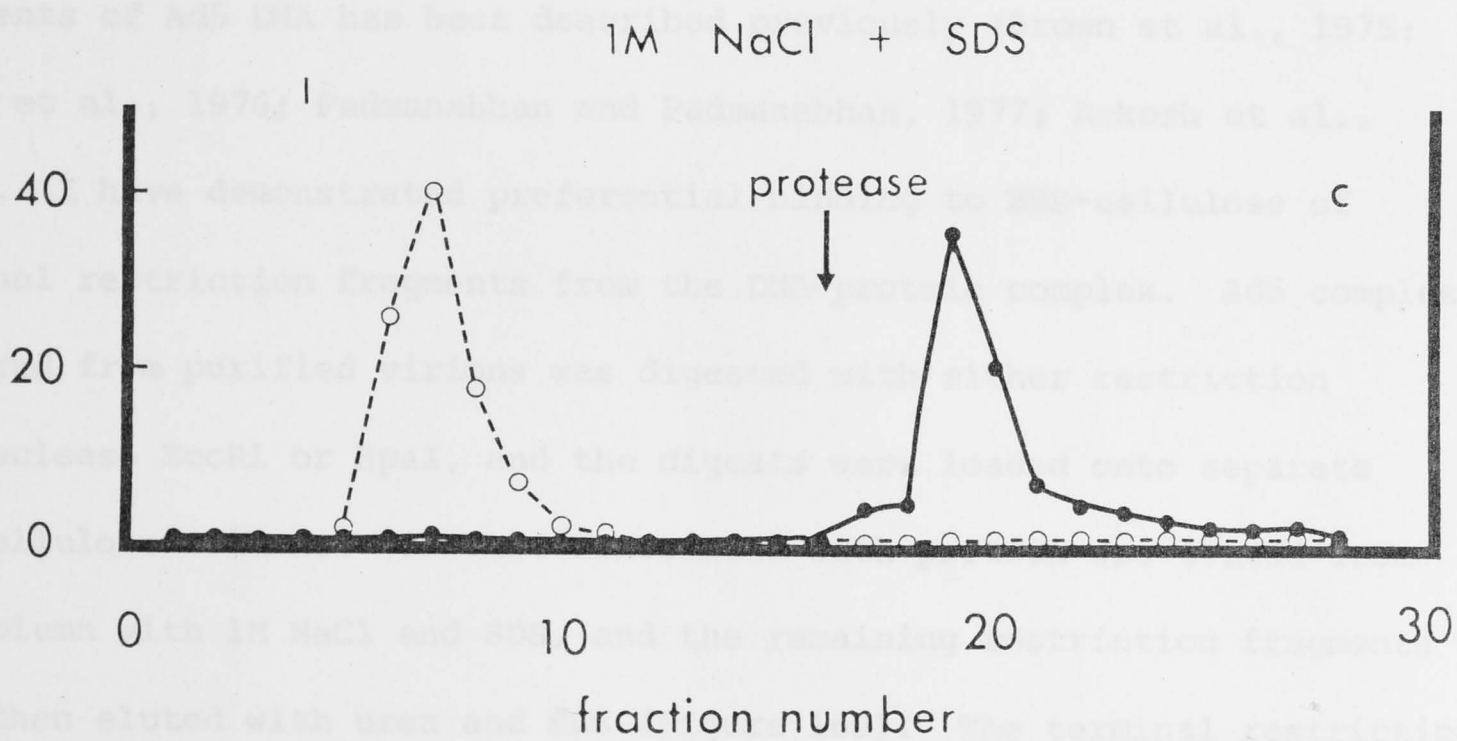
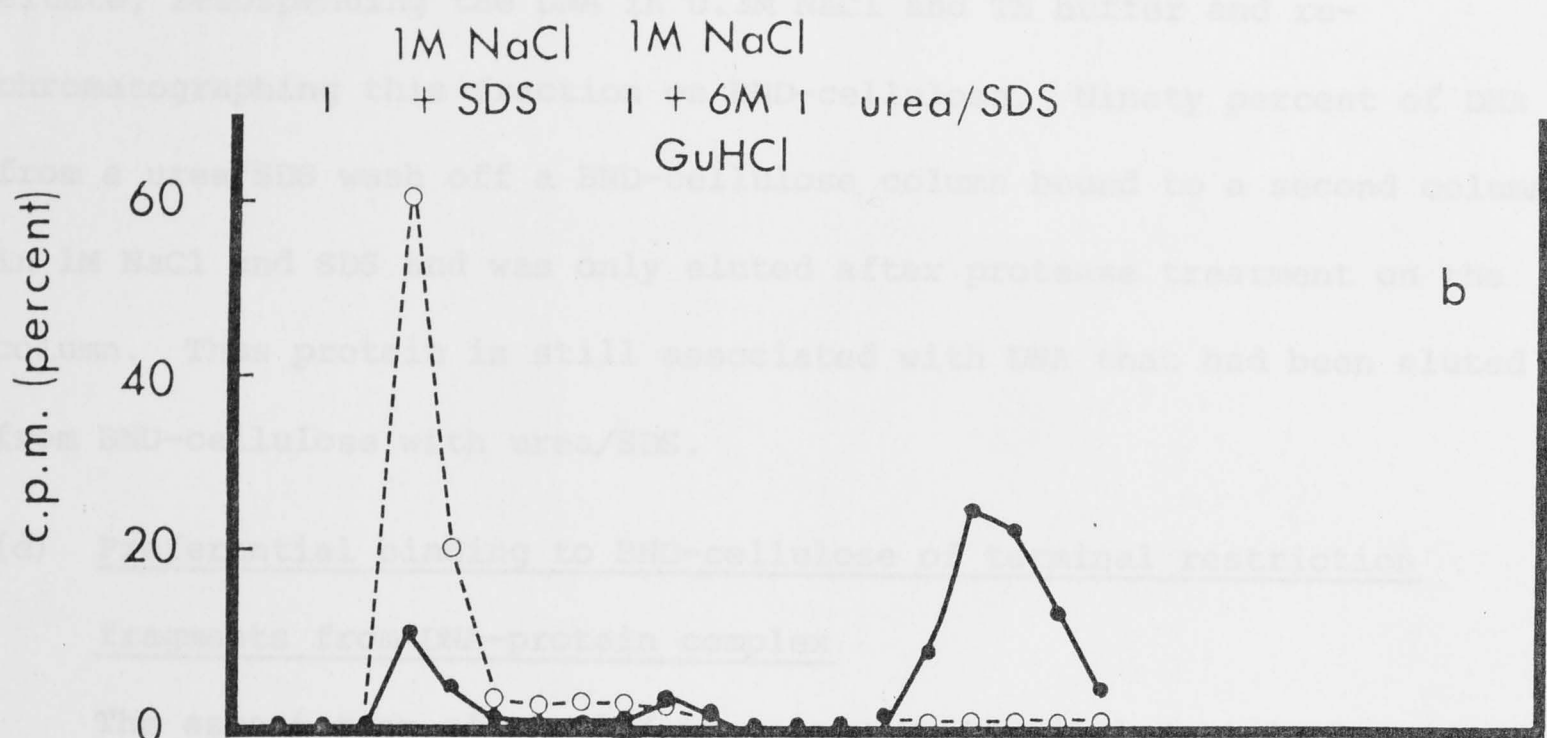
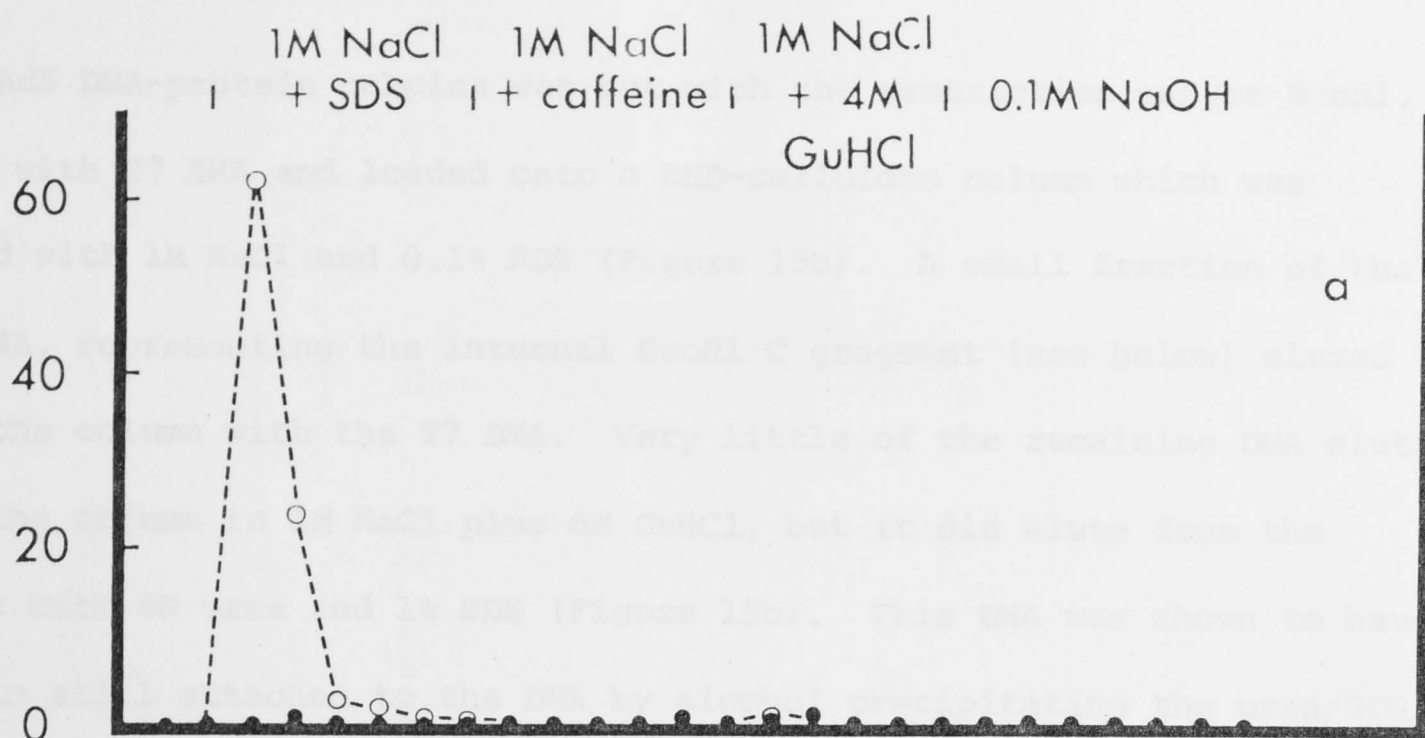
(c) Analysis of the DNA-protein complex on BND-cellulose

$^{32}\text{P}$ -labeled DNA-protein complex from Ad5, mixed with  $^3\text{H}$ -thymidine labeled T7 DNA, was loaded onto a BND-cellulose column in 0.3M NaCl and TE buffer (0.01M Tris, pH 8 and 0.01M EDTA) and the column was washed with TE buffer containing 1M NaCl and 0.1% SDS, and then with TE buffer containing 1M NaCl and 2% caffeine. Sodium dodecyl sulphate (SDS, 0.1%) was added to the buffers, as this addition increased the recovery of protein-free double-stranded DNA to 100%. Addition of SDS to the 0.3M NaCl loading buffer, prior to chromatography, prevented binding of the DNA-protein complex to the column. Figure 15a shows that  $^{32}\text{P}$  DNA-protein complex from Ad5 did not elute from the column with either 1M NaCl, which normally elutes double-stranded adenovirus DNA (Bellett and Younghusband, 1972; van der Eb, 1973). Further washing with 2% caffeine and 1M NaCl and 4M GuHCl, and with 0.1M NaOH, failed to elute Ad5 DNA-protein complex from the column. All of the double-stranded T7 DNA, which has a similar molecular weight to Ad5 DNA, eluted from the column in 1M NaCl buffer.

This result indicated that the protein moiety of the DNA-protein complex prevented DNA from eluting from the column, and this was confirmed by the following experiment: Ad5 DNA-protein complex was mixed with T7 DNA, loaded onto a BND-cellulose column and the column was washed with 1M NaCl and 0.1% SDS, which eluted 100% of the T7 DNA, but not the  $^{32}\text{P}$  Ad5 DNA-protein complex (Figure 15c). Protease VI (1 mg in 1 ml of the same buffer) was then applied to the column, which was incubated at 37°C for 30 minutes. The  $^{32}\text{P}$  Ad5 DNA was then completely eluted from the column by 1M NaCl and 0.1% SDS (Figure 15c).

Figure 15

BND-cellulose chromatography of Ad5 DNA-protein complex from purified virions.  $^{32}\text{P}$ -labeled Ad5 DNA-protein complex ( $\bullet\text{---}\bullet$ ) and  $^3\text{H}$ -labeled T7 DNA ( $\text{o}\text{-----}\text{o}$ ) were loaded onto a BND-cellulose column in 0.3M NaCl in TE buffer and the column was washed with 0.3M NaCl in TE buffer. DNA was eluted from the column with the indicated buffers. 0.1% SDS was used with 1M NaCl in TE buffer and 1% SDS was used with 8M urea in TE buffer. Caffeine was 2% and where indicated, the column was incubated in 1 mg/ml protease VI for  $\frac{1}{2}$  hr at 37°C and DNA eluted with the appropriate buffer. Results are expressed as radioactivity in each 0.5 ml fraction as a percentage of the total radioactivity added to the column. (a)  $^{32}\text{P}$ -labeled Ad5 DNA-protein complex (2,600 cpm) and  $^3\text{H}$ -labeled T7 DNA (66,000 cpm). (b) EcoRI digested  $^{32}\text{P}$  Ad5 DNA-protein complex (5,000 cpm) and  $^3\text{H}$ -labeled T7 DNA (66,000 cpm). (c)  $^{32}\text{P}$ -labeled Ad5 DNA-protein complex (2,900 cpm) and  $^3\text{H}$ -labeled T7 DNA (69,000 cpm).





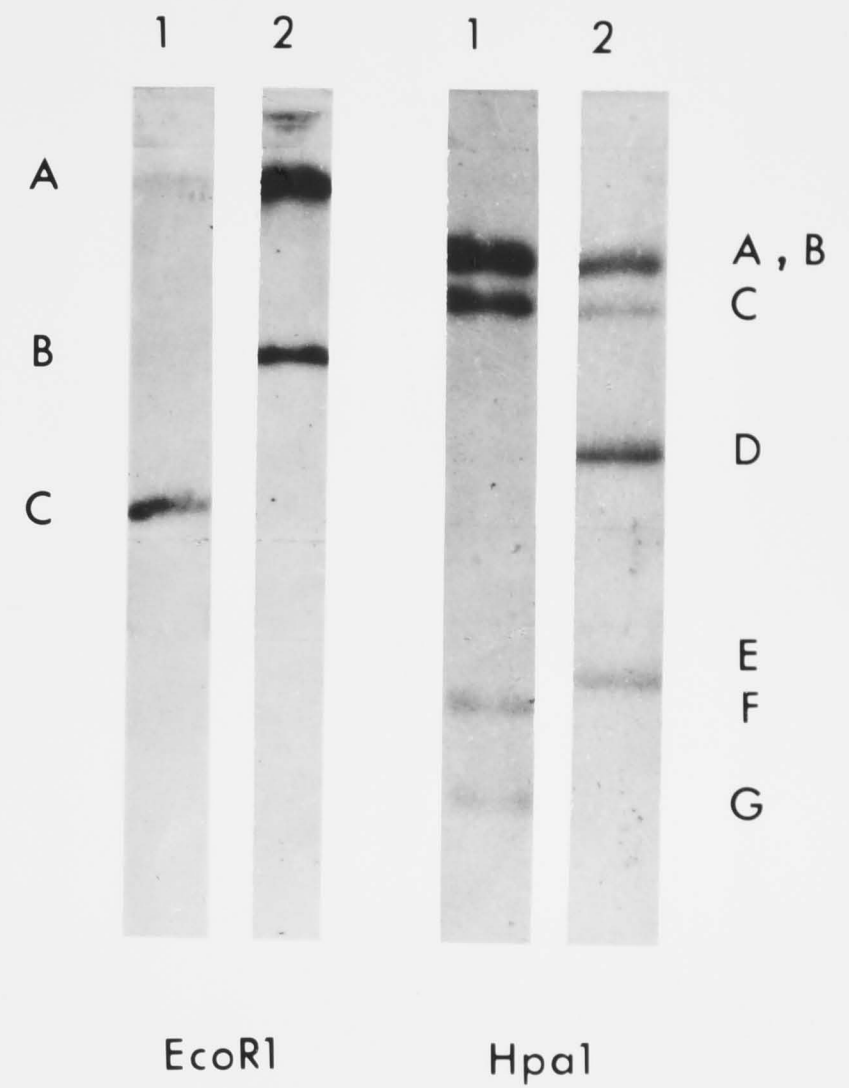
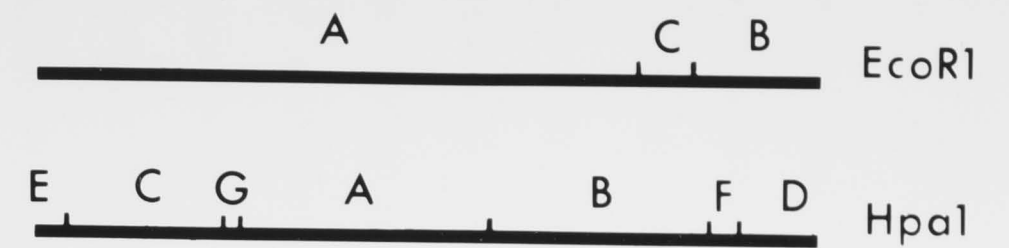
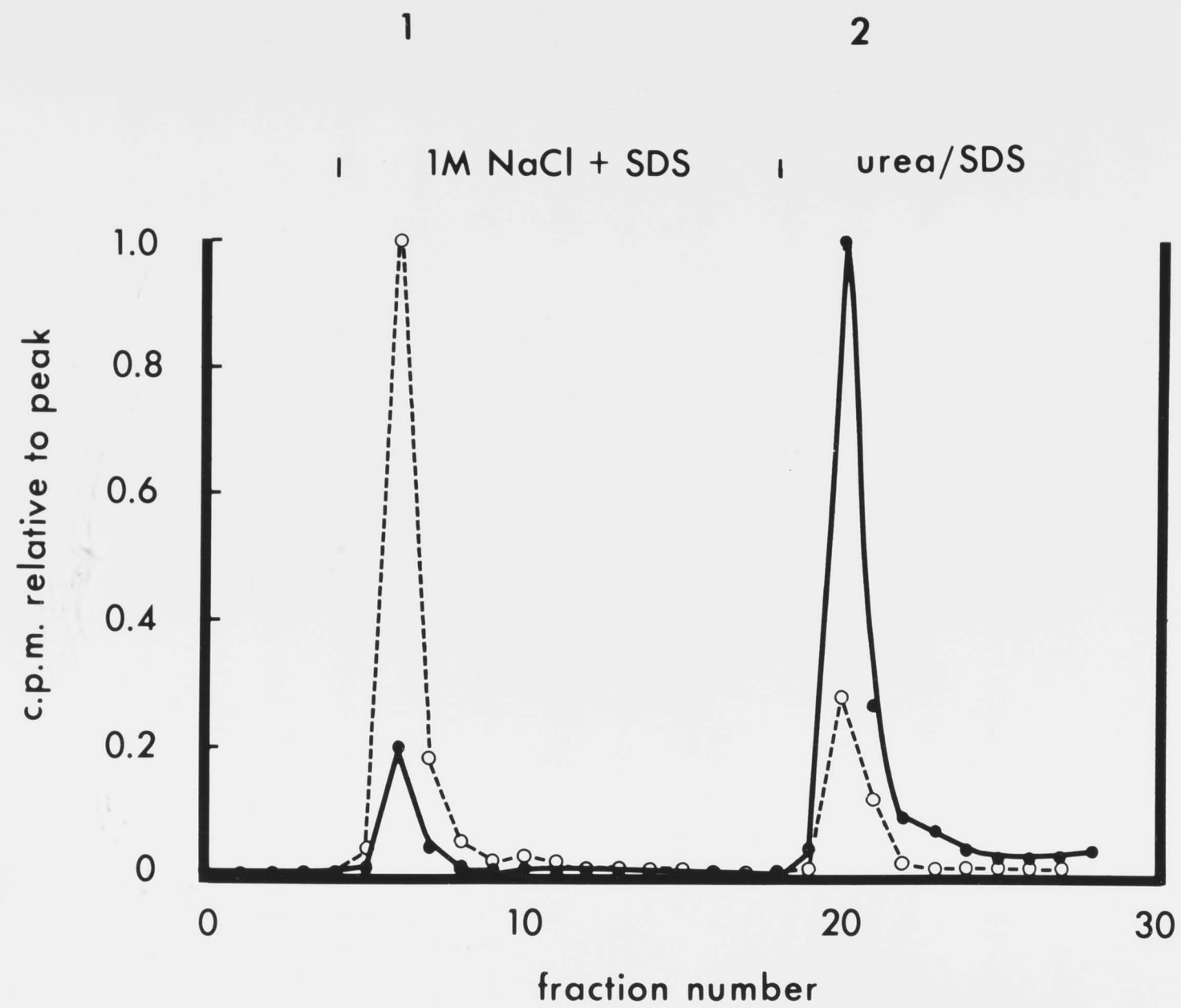
Ad5 DNA-protein complex was cut with the restriction enzyme EcoRI, mixed with T7 DNA and loaded onto a BND-cellulose column which was washed with 1M NaCl and 0.1% SDS (Figure 15b). A small fraction of the Ad5 DNA, representing the internal EcoRI C fragment (see below) eluted from the column with the T7 DNA. Very little of the remaining DNA eluted from the column in 1M NaCl plus 6M GuHCl, but it did elute from the column with 8M urea and 1% SDS (Figure 15b). This DNA was shown to have protein still attached to the DNA by alcohol precipitating the urea/SDS eluate, resuspending the DNA in 0.3M NaCl and TE buffer and re-chromatographing this fraction on BND-cellulose. Ninety percent of DNA from a urea/SDS wash off a BND-cellulose column bound to a second column in 1M NaCl and SDS and was only eluted after protease treatment on the column. Thus protein is still associated with DNA that had been eluted from BND-cellulose with urea/SDS.

(d) Preferential binding to BND-cellulose of terminal restriction fragments from DNA-protein complex

The association of the 55K protein with terminal restriction fragments of Ad5 DNA has been described previously (Brown et al., 1975; Sharp et al., 1976; Padmanabhan and Padmanabhan, 1977; Rekosh et al., 1977). I have demonstrated preferential binding to BND-cellulose of terminal restriction fragments from the DNA-protein complex. Ad5 complex prepared from purified virions was digested with either restriction endonuclease EcoRI or HpaI, and the digests were loaded onto separate BND-cellulose columns. DNA not associated with protein was eluted from the column with 1M NaCl and SDS, and the remaining restriction fragments were then eluted with urea and SDS (Figure 16a). The terminal restriction fragments of Ad5 DNA produced after digestion with endonuclease EcoRI comprise most of the DNA, whereas the reverse is true for Ad5 DNA digested

Figure 16

- (a) (on left) BND-cellulose chromatography of DNA-protein complex from purified virus.  $^{32}\text{P}$ -labeled Ad5 DNA-protein complex was digested with either EcoRI (●—●) (37,000 cpm) or HpaI (o-----o) (38,900 cpm), made 0.3M in NaCl and run on BND-cellulose with the indicated buffers.
- (b) (on right) The fragments from each wash were alcohol-precipitated, deproteinized and electrophoresed on 1% agarose. Lane 1: fragments from the 1M NaCl and SDS wash; Lane 2: fragments from the urea/SDS wash. The restriction map for Ad5 DNA with these enzymes and the fragment assignments are indicated (Mulder et al., 1974).





with endonuclease HpaI (Mulder et al., 1974; see maps in Figure 16b). This was reflected in the elution profiles of DNA digested with each enzyme (Figure 16a).

DNA from both the 1M NaCl/SDS and the urea/SDS washes was alcohol-precipitated, resuspended in 0.3M NaCl in TE buffer, treated with protease VI to remove protein and analysed by electrophoresis on a 1% agarose slab gel. Only internal restriction fragments from both EcoRI and HpaI digested DNA-protein complex were eluted in 1M NaCl and SDS (Figure 16b). The terminal fragments produced by both enzymes remained bound to BND-cellulose in 1M NaCl and were eluted with urea/SDS. Furthermore, terminal restriction enzyme fragments of Ad5 DNA-protein complex bound to BND-cellulose in 1M NaCl and SDS and could be eluted in this buffer after protease treatment on the column as shown in Figure 15c with whole DNA-protein complex. A small amount of internal HpaI restriction fragments were eluted with urea/SDS, but these internal fragments could be eluted without urea by more extensive washing in 1M NaCl plus 2% caffeine, without eluting the terminal fragments.

(e) Binding of adenovirus DNA is mediated by the terminal protein

DNA-protein complex purified from adenovirus virions by the method described in section 4.4(b) contains only the 55K protein attached to the ends of the DNA (Rekosh et al., 1977). This indicated that the binding of adenovirus DNA to BND-cellulose in the presence of 1M NaCl and SDS was due to the 55K terminal protein. However, I tested whether the presence of other adenovirus virion proteins either caused non-specific binding of de-proteinized Ad5 DNA to BND-cellulose or alternatively inhibited the binding of Ad5 DNA-protein complex to the column.

Adenovirus DNA, labeled with  $^{32}\text{P}$  and prepared from purified virions by disruption of the virus with 0.5% SDS, digestion of protein with protease VI and removal of peptides by phenol extraction, was mixed with  $^{35}\text{S}$ -labeled virion proteins.  $^{35}\text{S}$ -labeled virion proteins were prepared by disrupting  $^{35}\text{S}$ -labeled purified virus with 4M GuHCl in TE buffer and then either diluting the protein mix with TE buffer containing 0.3M NaCl (1 in 20 dilution), or removing GuHCl by dialysis against TE buffer containing 0.3M NaCl (both methods gave similar results). The Ad5 DNA and Ad5 virion proteins were loaded onto a BND-cellulose column in TE buffer containing 0.3M NaCl, and the column was washed with 1M NaCl, 0.1% SDS and 2% caffeine in TE buffer, followed by 8M urea and 1% SDS (Figure 17a). All of the Ad5 DNA eluted from the column with 1M NaCl and caffeine. However, all of the  $^{35}\text{S}$ -labeled adenovirus protein remained bound to the column in this buffer and only eluted with urea/SDS (Figure 17a). Thus, the Ad5 DNA did not bind non-specifically to the column in the presence of other virion proteins, which again suggests that the binding of the Ad5 DNA-protein complex is solely due to the covalently bound 55K terminal protein.

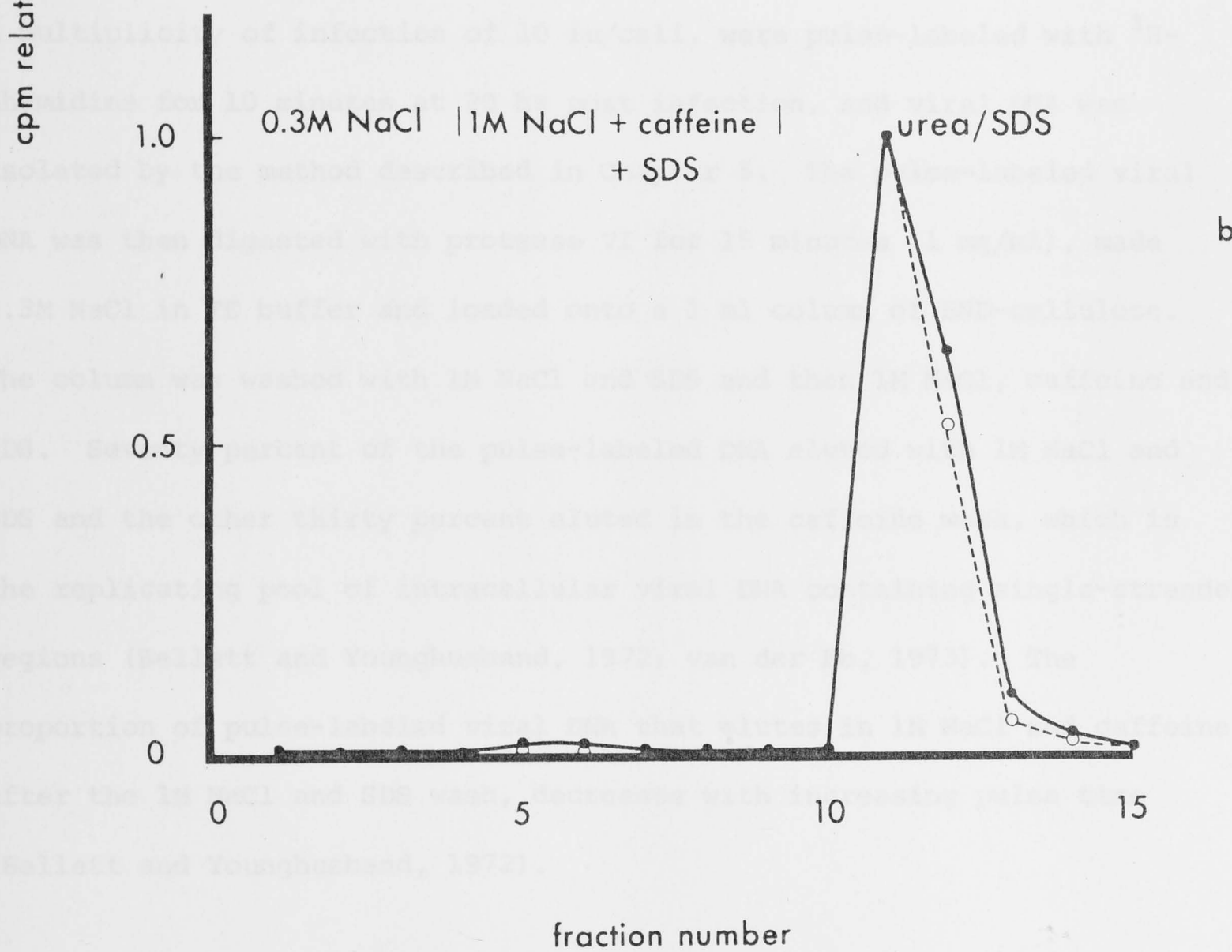
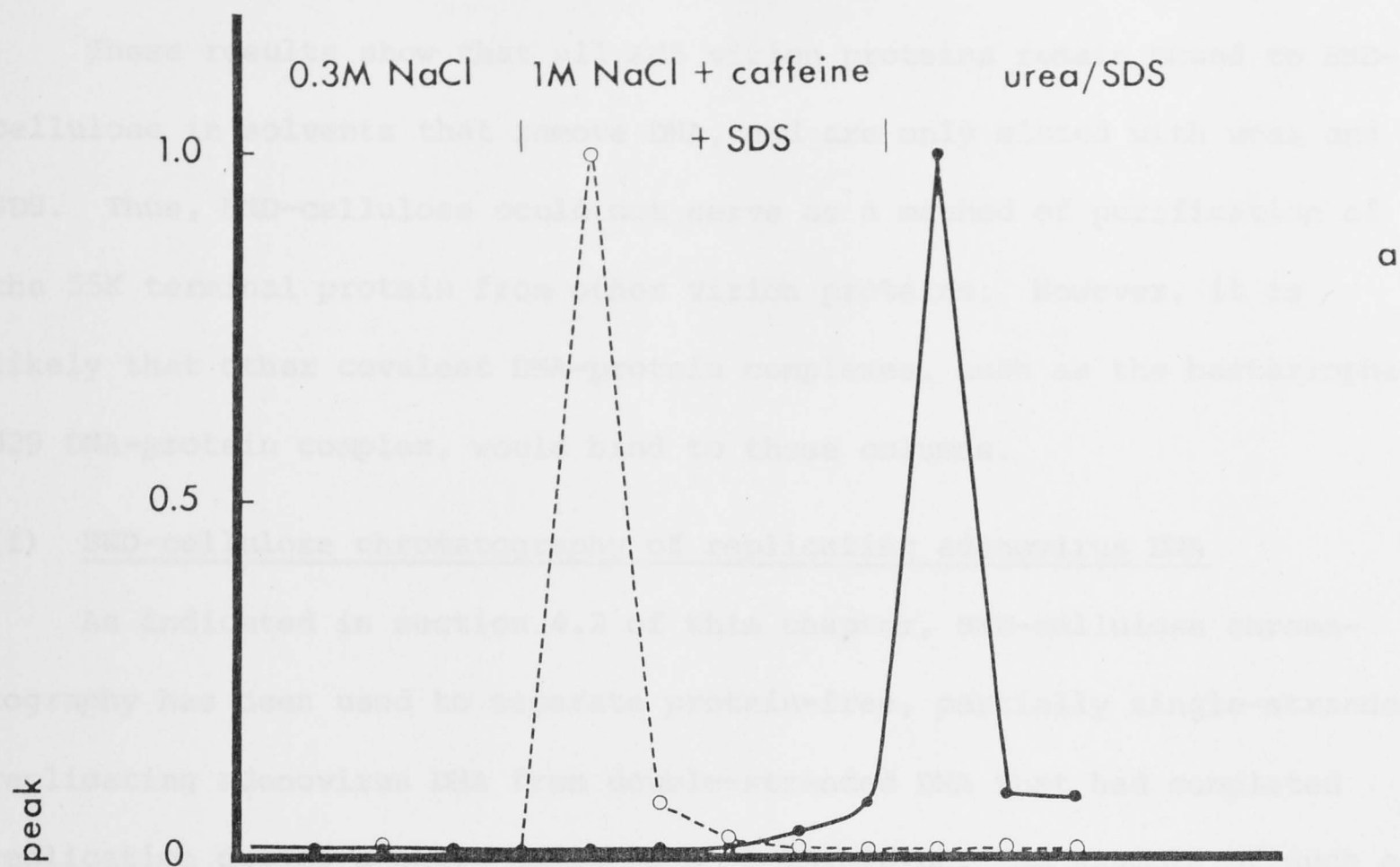
Figure 17(b) shows that when  $^3\text{H}$ -labeled Ad5 DNA-protein complex and  $^{35}\text{S}$ -labeled Ad5 virion proteins were co-chromatographed on BND-cellulose, the presence of other virion proteins did not inhibit the binding of the complex. Aggregation of adenovirus terminal protein and other virion proteins enabled Keegstra et al. (1977) to see protein 'knobs' on the termini of adenovirus DNA when viewed in the electron microscope. It is not known whether virion protein forms an aggregate with terminal protein under the conditions used in Figure 17b, but the complex of DNA and terminal protein was not affected in its chromatographic behaviour by the other proteins.

Figure 17

BND-cellulose chromatography of Ad5 virion protein, DNA and DNA-protein complex. (a) Chromatography of  $^{35}\text{S}$ -labeled Ad5 virion proteins (●—●) (300 cpm) and  $^{32}\text{P}$ -labeled, deproteinized Ad5 DNA (o-----o) (14,000 cpm). (b) Chromatography of  $^{35}\text{S}$ -labeled Ad5 virion proteins (●—●) (9,000 cpm) and  $^3\text{H}$ -thymidine labeled Ad5 DNA-protein complex (o-----o) (17,300 cpm). Fractions were 1 ml and radioactivity was determined in water-miscible scintillation fluid.

$^{35}\text{S}$ -labeled Ad5 virion proteins were prepared by disrupting purified virus with 4M GuHCl in TE buffer and then removing the GuHCl by dialysis against 0.3M NaCl and TE buffer.





These results show that all Ad5 virion proteins remain bound to BND-cellulose in solvents that remove DNA, and are only eluted with urea and SDS. Thus, BND-cellulose could not serve as a method of purification of the 55K terminal protein from other virion proteins. However, it is likely that other covalent DNA-protein complexes, such as the bacteriophage  $\phi$ 29 DNA-protein complex, would bind to these columns.

(f) BND-cellulose chromatography of replicating adenovirus DNA

As indicated in section 4.2 of this chapter, BND-cellulose chromatography has been used to separate protein-free, partially single-stranded replicating adenovirus DNA from double-stranded DNA that had completed replication during a short pulse with  $^3\text{H}$ -thymidine. An example of such a separation is shown in Figure 18. Human HEK cells, infected with Ad5 at a multiplicity of infection of 10 iu/cell, were pulse-labeled with  $^3\text{H}$ -thymidine for 10 minutes at 20 hr post infection, and viral DNA was isolated by the method described in Chapter 5. The pulse-labeled viral DNA was then digested with protease VI for 15 minutes (1 mg/ml), made 0.3M NaCl in TE buffer and loaded onto a 1 ml column of BND-cellulose. The column was washed with 1M NaCl and SDS and then 1M NaCl, caffeine and SDS. Seventy percent of the pulse-labeled DNA eluted with 1M NaCl and SDS and the other thirty percent eluted in the caffeine wash, which is the replicating pool of intracellular viral DNA containing single-stranded regions (Bellett and Younghusband, 1972; van der Eb, 1973). The proportion of pulse-labeled viral DNA that elutes in 1M NaCl and caffeine, after the 1M NaCl and SDS wash, decreases with increasing pulse time (Bellett and Younghusband, 1972).

Figure 18

BND-cellulose chromatography of pulse-labeled Ad5 DNA.

Ad5-infected HEK cells were pulse-labeled for 10 minutes with  $^3\text{H}$ -thymidine (100  $\mu\text{Ci/ml}$ ) and intracellular viral DNA was isolated as described in section 5.2. The DNA was treated with protease VI and made 0.3M in NaCl in TE buffer, then chromatographed on BND-cellulose with the indicated buffers. Fractions were 1 ml and radioactivity was determined in water-miscible scintillation fluid.



# 4.3 Discussion

The DNA-protein complex isolated from virus contains a

linear, double-stranded DNA molecule of 12.5 kb, which is

covalently linked to the protein core. The DNA molecule

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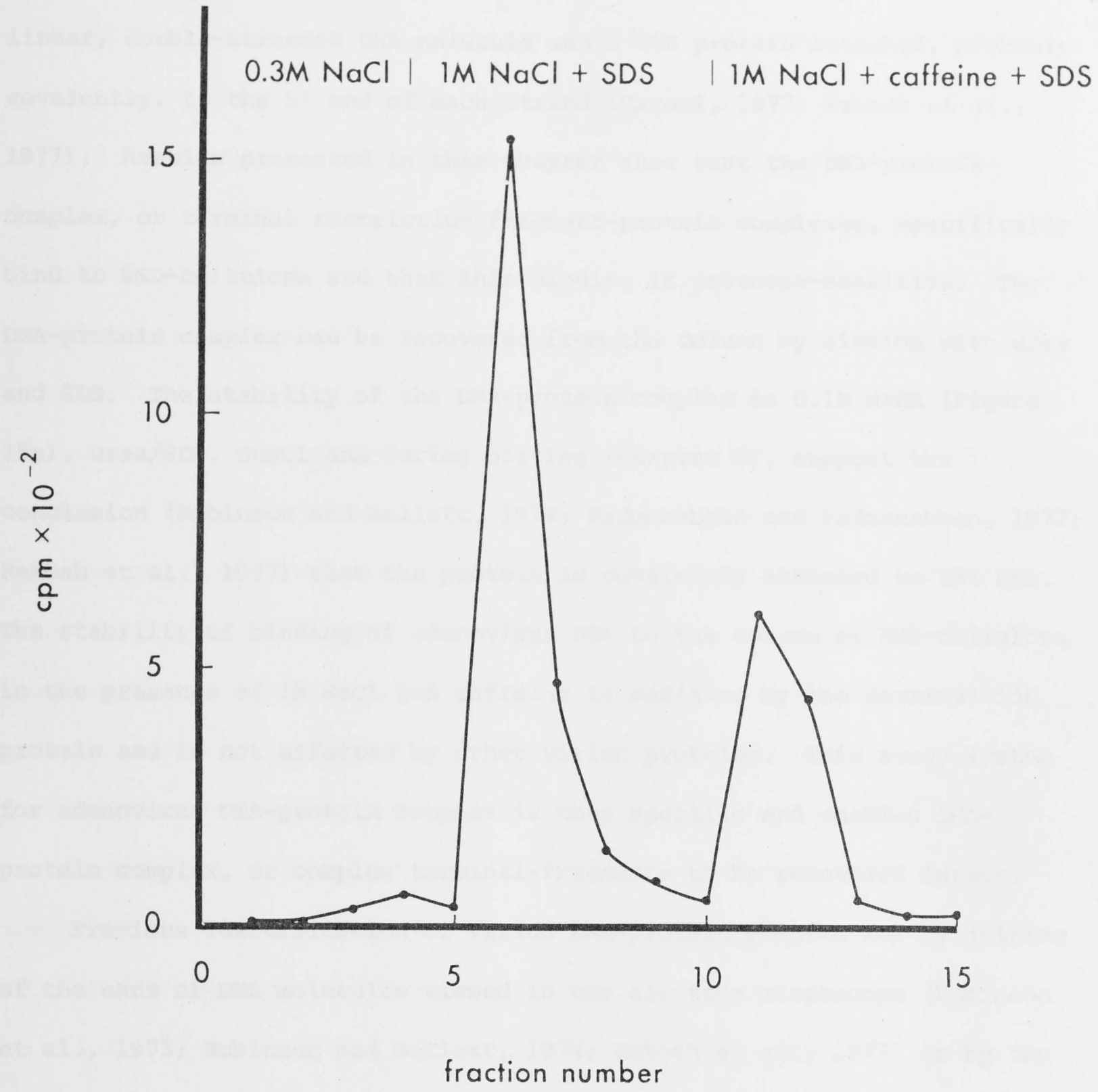
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protein complex. The DNA molecule is covalently linked to the



#### 4.5 Discussion

The DNA-protein complex isolated from Ad5 virions consists of a linear, double-stranded DNA molecule and a 55K protein attached, probably covalently, to the 5' end of each strand (Carusi, 1977; Rekosh et al., 1977). Results presented in this chapter show that the DNA-protein complex, or terminal restriction fragment-protein complexes, specifically bind to BND-cellulose and that this binding is protease-sensitive. The DNA-protein complex can be recovered from the column by elution with urea and SDS. The stability of the DNA-protein complex in 0.1M NaOH (Figure 15a), urea/SDS, GuHCl and during boiling (Chapter 5), support the conclusion (Robinson and Bellett, 1974; Padmanabhan and Padmanabhan, 1977; Rekosh et al., 1977) that the protein is covalently attached to the DNA. The stability of binding of adenovirus DNA to the column of BND-cellulose in the presence of 1M NaCl and caffeine is mediated by the terminal 55K protein and is not affected by other virion proteins. This assay system for adenovirus DNA-protein complex is thus specific and enables DNA-protein complex, or complex terminal-fragments to be recovered intact.

Previous identification of virion DNA-protein complex was by joining of the ends of DNA molecules viewed in the electron microscope (Robinson et al., 1973; Robinson and Bellett, 1974; Rekosh et al., 1977) or by the inability of DNA-protein complex to enter agarose gels (Brown et al., 1975; Sharp et al., 1976; Padmanabhan and Padmanabhan, 1977; H.B. Younghusband, personal communication). Keegstra et al. (1977) have observed 'knobs' of protein on the termini of adenovirus DNA in the electron microscope; however, these were likely to be protein aggregates of the 55K protein and other virion proteins.

During the course of this work, filter binding assays for adenovirus DNA-protein complex have been developed (D.H. Coombs and G.D. Pearson,

personal communication; Padmanabhan and Padmanabhan, 1977). However, in the latter case, only 80% to 90% binding of DNA-protein complex to filters was achieved. The filter binding assay for Ad2 DNA-protein complex on glass fibre filters (D.H. Coombs and G.D. Pearson, personal communication) is salt-dependent, as are the binding assays for non-covalent DNA-protein interactions (Jones and Berg, 1966; Riggs et al., 1970). Neither of the filter binding assays is capable of distinguishing between replicating and completed DNA-protein complex molecules isolated after a short pulse of  $^3\text{H}$ -thymidine. Recently, M.S. Horwitz and L.M. Kaplan (personal communication) have also observed binding of adenovirus DNA-protein complex to columns of BND-cellulose.

The binding of adenovirus DNA-protein complex to BND-cellulose was assayed by detection of label in the DNA moiety. This was done for two reasons, firstly because isolation of in vivo  $^{35}\text{S}$  labeled 55K terminal protein is extremely difficult, so much so that Rekosh et al. (1977) labeled the protein in vitro with  $^{125}\text{I}$  to obtain a molecular weight for the protein. Secondly, an assay for DNA-protein complex with label in the DNA moiety will enable analysis of pulse-labeled intracellular viral DNA for the presence of terminal protein.

All adenovirus virion proteins bind to BND-cellulose and can be quantitatively recovered from the column by elution with urea and SDS. This binding of proteins suggests that other covalently linked DNA-protein complexes could be assayed on BND-cellulose in a similar way to the adenovirus DNA-protein complex. These complexes include the DNA-protein complexes from the *Bacillus subtilis* phages, the SV40 DNA-protein complex, the plasmid relaxation complexes, the  $\phi\text{X174}$ -cisa complex and the topoisomerase enzyme complexed to DNA (see section 1.5). Different proteins have also been shown to bind to nitrocellulose filters (Yarus



and Berg, 1970) which may indicate that the cellulose part of the BND-cellulose may bind the proteins.

Digestion of Ad5 DNA-protein complex with protease VI on the BND-cellulose column allows the DNA moiety to be eluted from the column with the usual elution buffers of either 1M NaCl or 1M NaCl and caffeine (section 4.2). BND-cellulose chromatography has already been shown to separate completed and replicating protein-free DNA molecules of intracellular adenovirus DNA because of the extensive single-stranded regions in replicating DNA (Bellett and Younghusband, 1972; van der Eb, 1973), and an example of such a separation is shown in Figure 18. Thus, an advantage of the BND-cellulose assay system is that DNA-protein complex isolated from infected cells will bind quantitatively to the column because of the protein moiety, and after protease treatment the DNA moiety can be separated into completed and replicating fractions. Pulse-labeled DNA can therefore be analysed both for its association with terminal protein and for its content of replicating molecules on the one column. Experiments of this nature are described in the next chapter.

An adenovirus is a virus that consists of a linear DNA molecule with terminal repetitions of unique sequences. The genome is approximately 35,000 (base) pairs long and ends. The terminal DNA is a protein coat that is very dense.

Replication of adenovirus DNA each strand, with the end of the DNA. Suspected, the 1977: Evidence for

CHAPTER 5

DNA replication. and Kelly, 1977: The

the protein of A protein associated with the ends of replicating adenovirus DNA molecules

(1977) concluded that DNA replication of replicating DNA Evidence suggested that self-complementary presented Chapter

In order to understand in adenovirus terminal protein (Chapter 4) proteins is associated with adenovirus DNA

## 5.1 Introduction

As discussed in previous chapters, the adenovirus chromosome consists of a linear, double-stranded DNA molecule with an inverted terminal repetition of approximately 100 base pairs in length and a 55,000 (55K) molecular weight protein covalently attached to each 5' end. The functions of the inverted terminal repetition and the 55K protein have yet to be determined.

Replication of adenovirus DNA proceeds in the 5' to 3' direction on each strand, with the origins and termini of replication at or near each end of the linear molecules (Horwitz, 1976; Weingärtner et al., 1976; Sussenbach and Kuijk, 1977; Ariga and Shimojo, 1977; Lechner and Kelly, 1977; Sussenbach and Kuijk, 1978). Approximately 20% of replicating DNA is single-stranded as a result of a strand displacement mechanism for DNA replication (Sussenbach et al., 1974; Levine et al., 1976; Lechner and Kelly, 1977; Kedinger et al., 1978). However, there still remains the problem of how replication at the 5' ends of linear adenovirus DNA is primed (Bellett and Younghusband, 1972; Chapter 3). Rekosh et al. (1977) considered this problem when they proposed a model for adenovirus DNA replication which involved priming of each newly synthesized 5' end of replicating DNA by a nucleotide attached to the 55K terminal protein. Evidence inconsistent with an alternative model based on priming by a self-complementary hairpin loop on the ends of the DNA has already been presented (Chapter 3, see also Sussenbach and Kuijk, 1978b).

In order to examine the role, if any, of the 55K terminal protein in adenovirus DNA replication, an assay system for the presence of the terminal protein on both mature and replicating DNA was developed (Chapter 4). In this chapter, evidence is presented which shows that a protein is associated with the ends of both completed and replicating adenovirus intracellular DNA. Evidence is also presented that this protein



is made prior to or at the onset of adenovirus DNA replication, consistent with the proposal that the terminal protein is involved in adenovirus DNA replication.

## 5.2 Methods

The following methods were used specifically for work reported in this chapter. Other general methods are described in Chapter 2.

### (a) Purification of $^3\text{H}$ -thymidine pulse-labeled Ad5 DNA-protein complex

The following procedure was designed to avoid proteolytic activity during DNA isolation: Confluent monolayers of HEK cells in 20 cm<sup>2</sup> petri dishes were infected with Ad5 in a multiplicity of infection of 10 pfu/cell and at 16-20 hr post infection (p.i.) were pulse-labeled for various times with 100  $\mu\text{Ci/ml}$   $^3\text{H}$ -thymidine (40-50 Ci/mMole) in Eagle's medium without calf serum. When required, infected cells were preincubated in medium containing 30  $\mu\text{g/ml}$  cycloheximide as indicated in the text. Cells were washed twice with ice-cold phosphate-buffered saline and a 1 ml solution containing 4M guanidine hydrochloride and 0.2% sarkosyl in STE was added at 4°C and left for at least 2 hr to lyse the cells. The cell lysate was layered onto a 5-20% neutral sucrose gradient containing 4M guanidine hydrochloride and 0.2% sarkosyl in STE and centrifuged in a Spinco SW27 rotor at 20,000 rpm for 16 hr at 4°C. Fractions were collected from the top by the use of an 'Auto-densi Flow' (Nuclear-Chicago, Buchler Instruments Division) and a sample of each fraction was removed for determination of acid-insoluble radioactivity. The viral DNA peak, including faster sedimenting replicative DNA, was pooled and dialysed against STE containing 0.2% sarkosyl, then further purified and concentrated on a CsCl equilibrium gradient containing 4M guanidine hydrochloride and 0.2% sarkosyl. The DNA-protein complex was then dialysed against STE for 16 hr at 4°C.

(b) Purification of  $^3\text{H}$ -thymidine labeled cell DNA

HEK cells were grown to 80% confluence in 20 cm<sup>2</sup> plastic petri dishes, labeled with  $^3\text{H}$ -thymidine (3  $\mu\text{Ci/ml}$ ) in 'Autopow' plus 10% calf serum and then lysed in 4M GuHCl and 0.2% sarkosyl in STE as for isolation of viral DNA. The cell DNA was purified by centrifugation in 5-20% neutral sucrose containing 4M GuHCl and 0.2% sarkosyl in STE (SW27, 25,000 rpm at 4°C for 2 hr) and then by recentrifugation in a CsCl/GuHCl/sarkosyl equilibrium gradient as for viral DNA.

(c) Staining of infected cells with fluorescent antibody

HEK cells were grown on glass coverslips in 20 cm<sup>2</sup> petri dishes, some were infected, and incubated for 20 hr with or without cycloheximide treatment as indicated. Infected and control cells were then fixed in acetone at -20°C, incubated with either rabbit antiserum against purified virus (V) or against early antigens (P; Russell et al., 1967), washed, stained with fluorescein-conjugated goat anti-rabbit IgG (Microbiological Associates), washed, and observed and photographed in a Leitz Orthomat ultra-violet fluorescence microscope.

### 5.3 Results

(a) Isolation of pulse-labeled Ad5 DNA from infected cells without protease treatment

The usual methods for isolating pulse-labeled viral DNA from infected cells involve protease digestion of the cell lysate and selective precipitation of the cell DNA with SDS by the procedure of Hirt (1967). This method would remove any protein associated with replicating DNA. I was concerned that during extraction of the DNA by the Hirt procedure without protease treatment, the replicating viral DNA-protein complex would aggregate and sediment with the cell DNA.

A different method was therefore used. Ad5-infected cells were pulse-labeled with  $^3\text{H}$ -thymidine at various times post infection and lysed at  $4^\circ\text{C}$  with 4M GuHCl and 0.2% sarkosyl in STE for 1-2 hr. The whole cell lysate was then layered onto a 5-20% neutral sucrose gradient in the same buffer at  $4^\circ\text{C}$  and centrifuged so that viral DNA sedimented half way down the gradient (Figure 19a), and was separated from non-covalently bound protein and cell DNA. The viral DNA, including both mature and replicating molecules, was then centrifuged in a CsCl/GuHCl/sarkosyl equilibrium gradient (Figure 19b) and the resulting DNA preparation was dialysed against STE at  $4^\circ\text{C}$  and used for BND-cellulose chromatography. Sometimes the pulse-labeled viral DNA preparation was concentrated by evaporation under a stream of nitrogen before use.

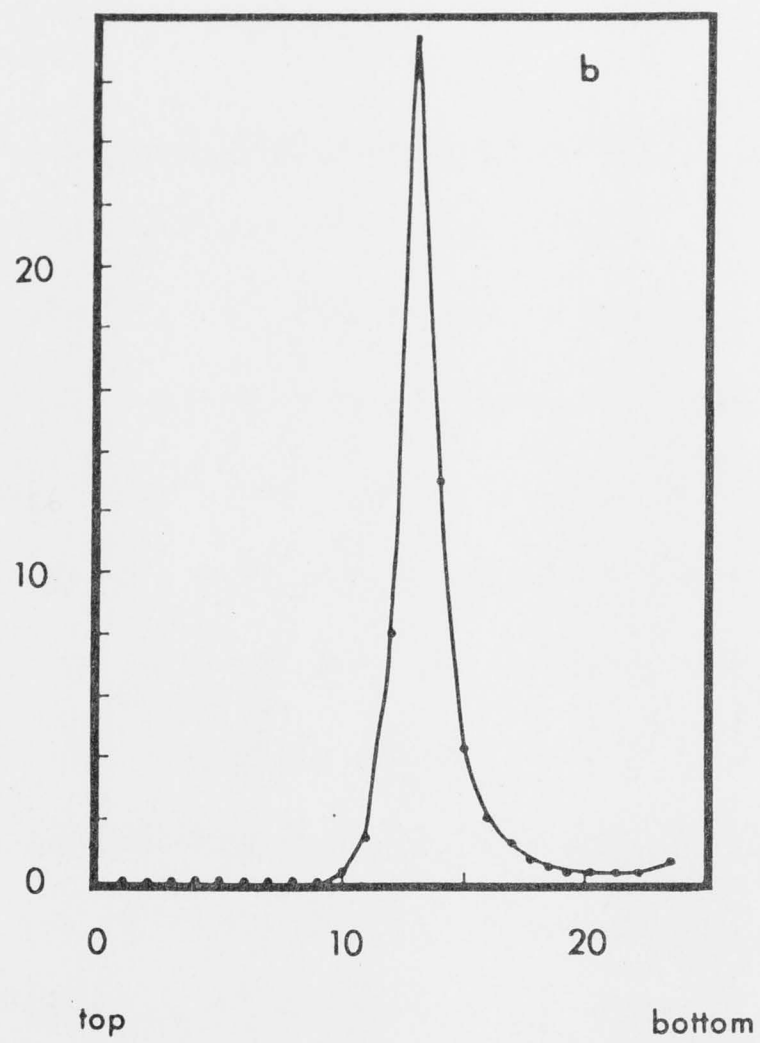
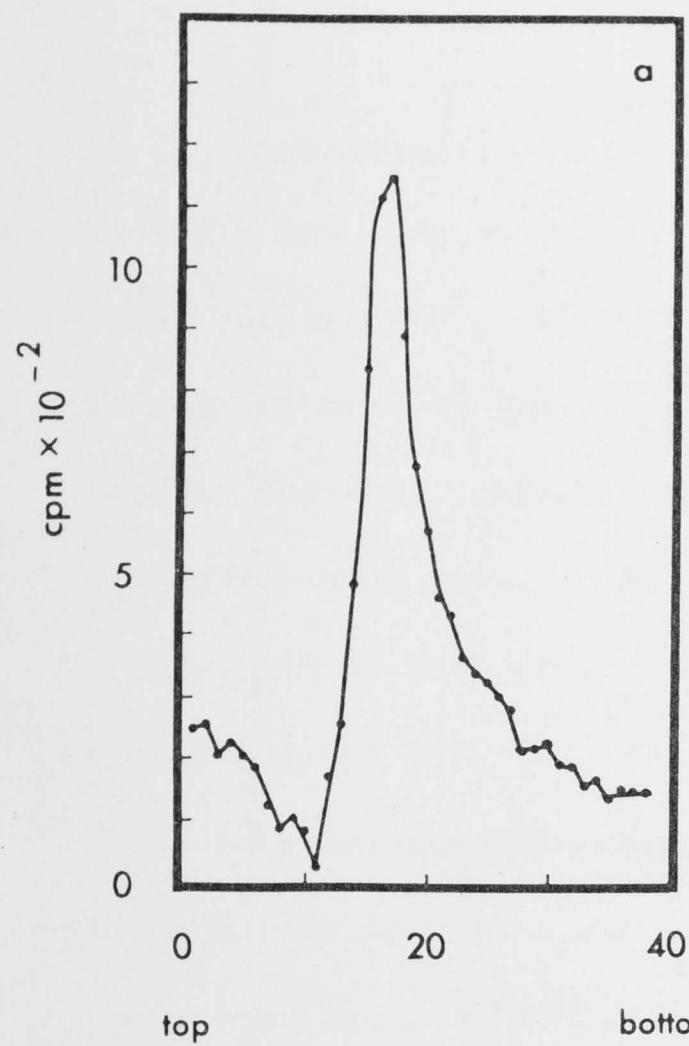
(b) Analysis of pulse-labeled DNA-protein complex on BND-cellulose

Viral DNA, pulse-labeled with  $^3\text{H}$ -thymidine for 10 minutes at 20 hr post infection and isolated without protease digestion as described above, did not elute from a BND-cellulose column with 1M NaCl and either SDS or caffeine (Figure 20b). In a number of similar experiments, 95-100% of pulse-labeled DNA bound to BND-cellulose columns and could be eluted only after treatment with protease. Both completed molecules (eluted by 1M NaCl) and replicating molecules of Ad5 DNA (which are partly single-stranded and require caffeine for elution; Bellett and Younghusband, 1972; van der Eb, 1973, see Chapter 4.2) behave as DNA-protein complex on BND-cellulose. These were identified after protease digestion of complex on the column (Figure 20b). Cell DNA isolated from uninfected HEK cells by a similar procedure eluted from BND-cellulose as expected for mature and replicating DNA free of bound protein (Figure 20a), indicating that non-specific association of protein with DNA during the isolation procedure was not responsible for the protease-sensitive binding of viral DNA to the column.



Figure 19

Isolation of pulse-labeled viral DNA-protein complex from infected cells. Ad5-infected cells were pulse-labeled for 10 minutes with  $^3\text{H}$ -thymidine at 20 hr post infection, then lysed in  $\text{GuHCl}$ /sarkosyl. The cell lysate was layered onto a 5-20% sucrose gradient containing  $\text{GuHCl}$  and sarkosyl and centrifuged for 16 hr at 20,000 rpm in an SW27 rotor at  $4^\circ\text{C}$  (a, sedimentation left to right). (b), the viral DNA peak was pooled (fractions 13 to 22) and dialysed against STE and sarkosyl and centrifuged in a  $\text{CsCl}$  gradient containing  $\text{GuHCl}$  and sarkosyl as described in Chapter 2. The viral peak (fractions 12 to 15) was dialysed against STE for BND-cellulose chromatography.



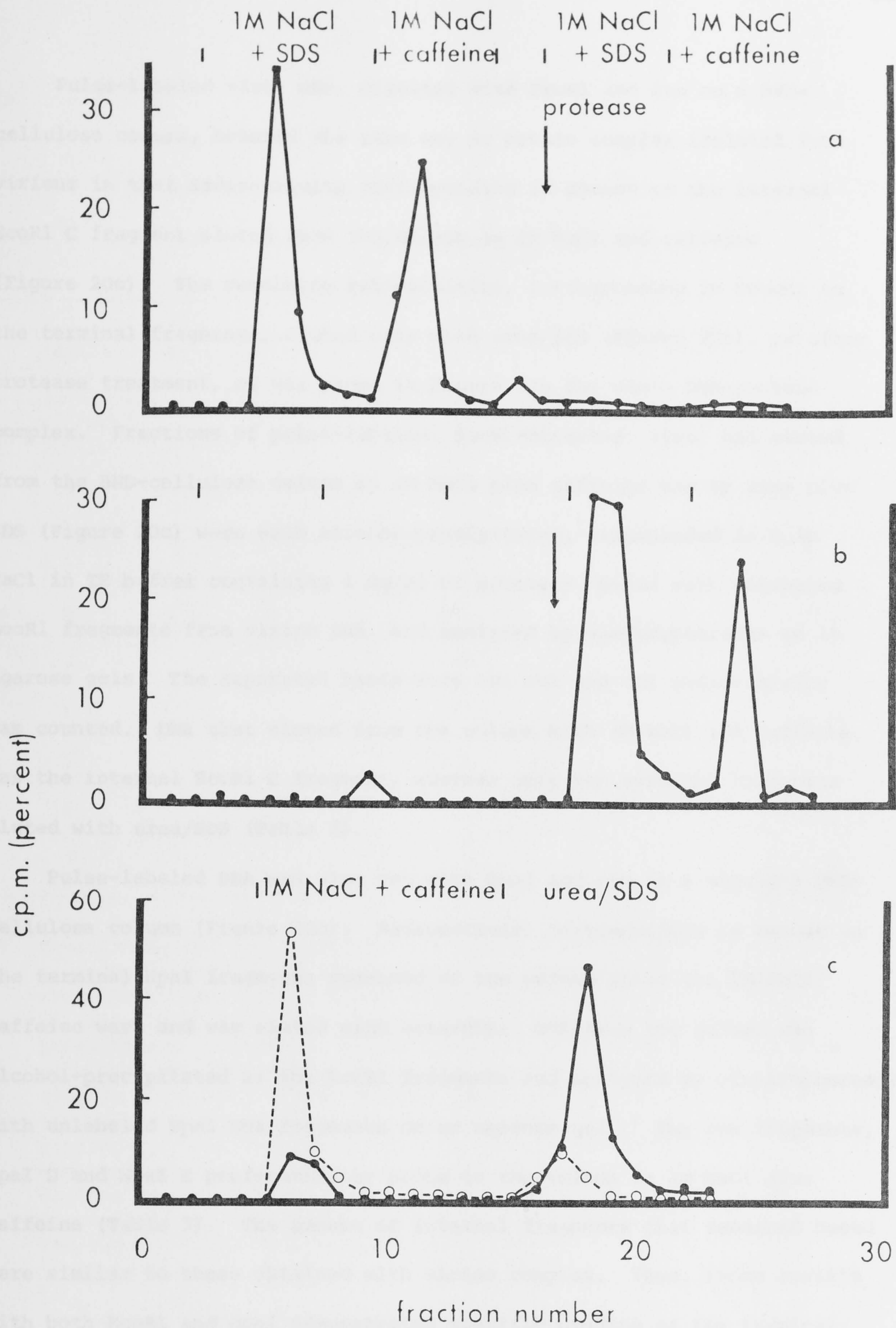
fraction number

Figure 20

BND-cellulose chromatography of replicating cell and viral DNA isolated in the absence of protease digestion.  $^3\text{H}$ -thymidine-labeled cell or viral DNA was loaded onto BND-cellulose columns in TE buffer containing 0.3M NaCl and washed with that buffer. DNA was eluted with the indicated buffers. Both (a) and (b) were washed with the same buffers and treated with protease as described in Figure 15.

(a) Cell DNA from uninfected HEK cells labeled with  $^3\text{H}$ -thymidine for 16 hr (11,500 cpm); (b) Ad5 DNA pulse-labeled for 10 minutes at 20 hr post infection (6,400 cpm); (c) Ad5 DNA pulse-labeled for 10 minutes at 20 hr post infection, was digested with either EcoRI (●—●) (6,800 cpm) or HpaI (o-----o) (57,000 cpm) and run on separate BND-cellulose columns. Results are expressed as the radioactivity in each 1 ml fraction as a percentage of the total radioactivity added to the column.





Pulse-labeled viral DNA, digested with EcoRI and run on a BND-cellulose column, behaved the same way as mature complex isolated from virions in that radioactivity corresponding in amount to the internal EcoRI C fragment eluted from the column in 1M NaCl and caffeine (Figure 20c). The remaining radioactivity, corresponding in amount to the terminal fragments, eluted only with urea/SDS (Figure 20c), or after protease treatment, as was shown in Figure 20a for whole DNA-protein complex. Fractions of pulse-labeled, EcoRI-digested, viral DNA eluted from the BND-cellulose column by 1M NaCl plus caffeine and by urea plus SDS (Figure 20c) were each alcohol-precipitated, resuspended in 0.3M NaCl in TE buffer containing 1 mg/ml of protease, mixed with unlabeled EcoRI fragments from virion DNA, and analysed by electrophoresis in 1% agarose gels. The separated bands were cut out and the radioactivity was counted. DNA that eluted from the column with 1M NaCl and caffeine was the internal EcoRI C fragment, whereas only the terminal fragments eluted with urea/SDS (Table 3).

Pulse-labeled DNA was also cut with HpaI and run on a separate BND-cellulose column (Figure 20c). Radioactivity corresponding in amount to the terminal HpaI fragments remained on the column after the 1M NaCl/caffeine wash and was eluted with urea/SDS. DNA from the column was alcohol-precipitated as for EcoRI fragments and analysed by electrophoresis with unlabeled HpaI DNA fragments on an agarose gel. The end fragments, HpaI D and HpaI E preferentially bound to the column in 1M NaCl plus caffeine (Table 3). The amount of internal fragments that remained bound were similar to those obtained with virion complex. Thus, these results with both EcoRI and HpaI demonstrated specific binding of the terminal restriction fragments of replicating and completed intracellular viral DNA-protein complex to BND-cellulose, and showed that protein was associated with newly replicated DNA within at least 4% of the end of the DNA.

Table 3

Radioactivity in separated EcoRI and HpaI fragments of  
replicating DNA after BND-cellulose chromatography<sup>(a)</sup>

Restriction fragment		cpm in 10 min pulse-labeled DNA	
		1	2
EcoRI	A	2	1,049
	B	0	570
	C	98	0
HpaI	A, B	19,492	1,562
	C	6,464	766
	D	169	4,165
	E	686	1,135
	F	846	124
	G	564	53

- (a) Replicating DNA was digested with either EcoRI or HpaI and chromatographed separately on BND-cellulose as shown in Figure 20c. DNA from the 1M NaCl and caffeine eluate (1) and the urea/SDS eluate (2) from each column was alcohol-precipitated, digested with protease and analysed by electrophoresis in agarose tube gels with unlabeled Ad5 DNA marker fragments. Gels were stained with ethidium bromide and each visible band was cut out of the gel and its radioactivity determined. In a number of similar experiments with HpaI, I found contamination of counts from HpaI F into the HpaI E fragment, resulting in apparently high HpaI E fragment eluting in the caffeine wash. Background counts have been subtracted and were obtained by counting the radioactivity in part of the gel which did not contain marker DNA.

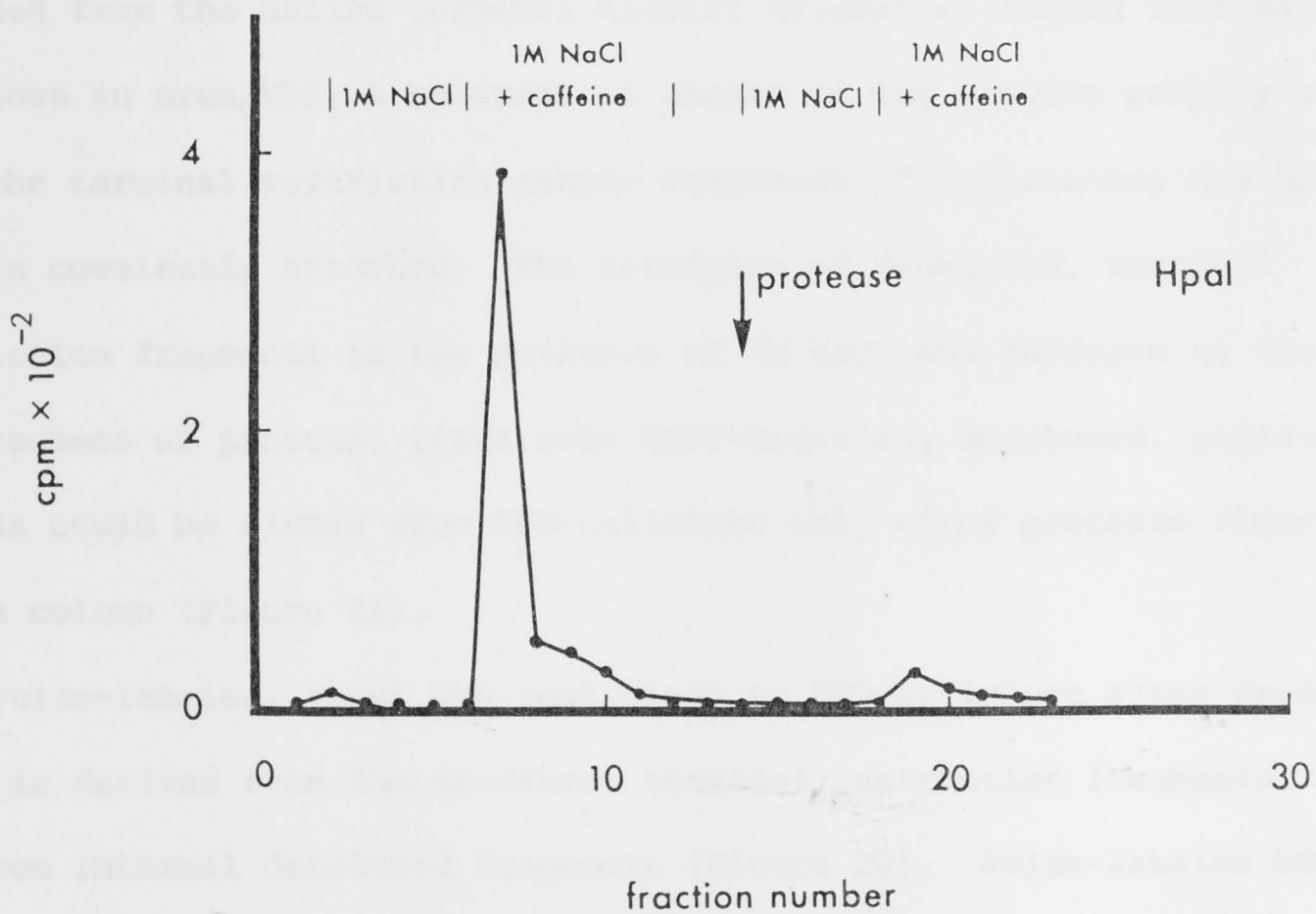
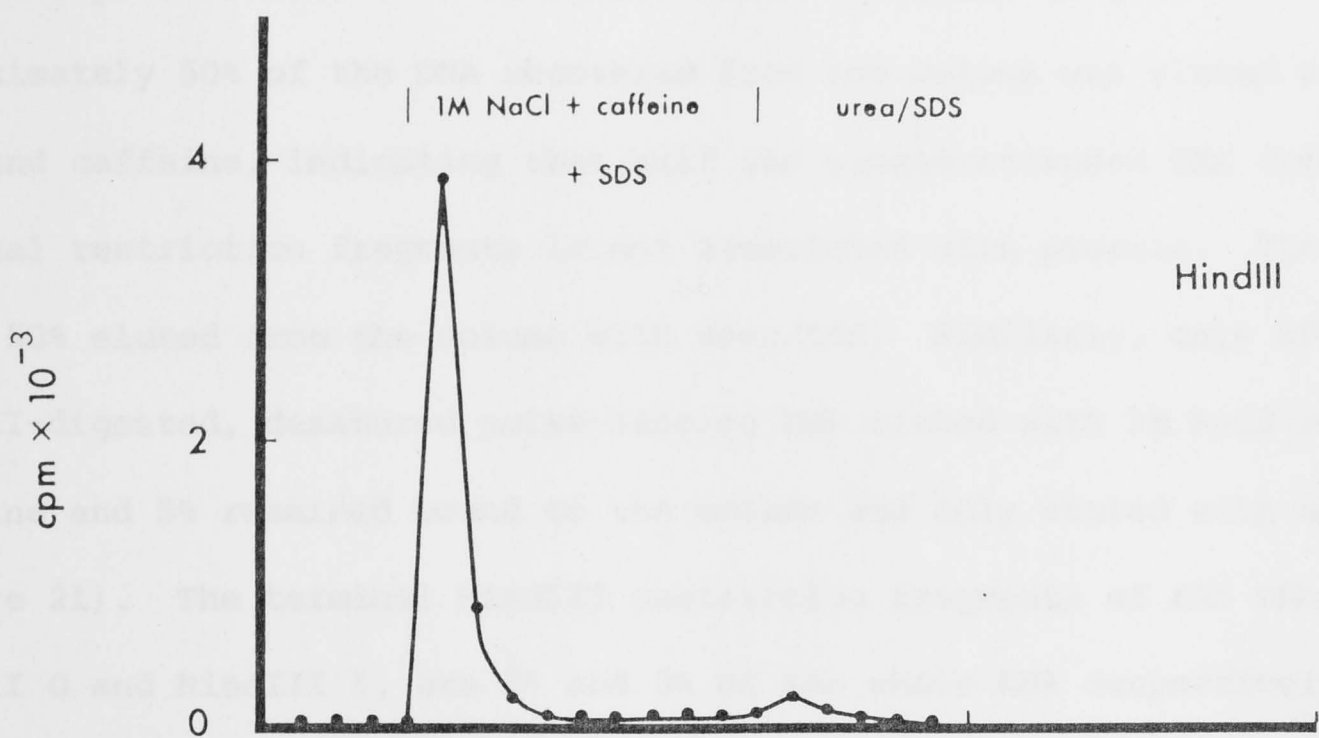
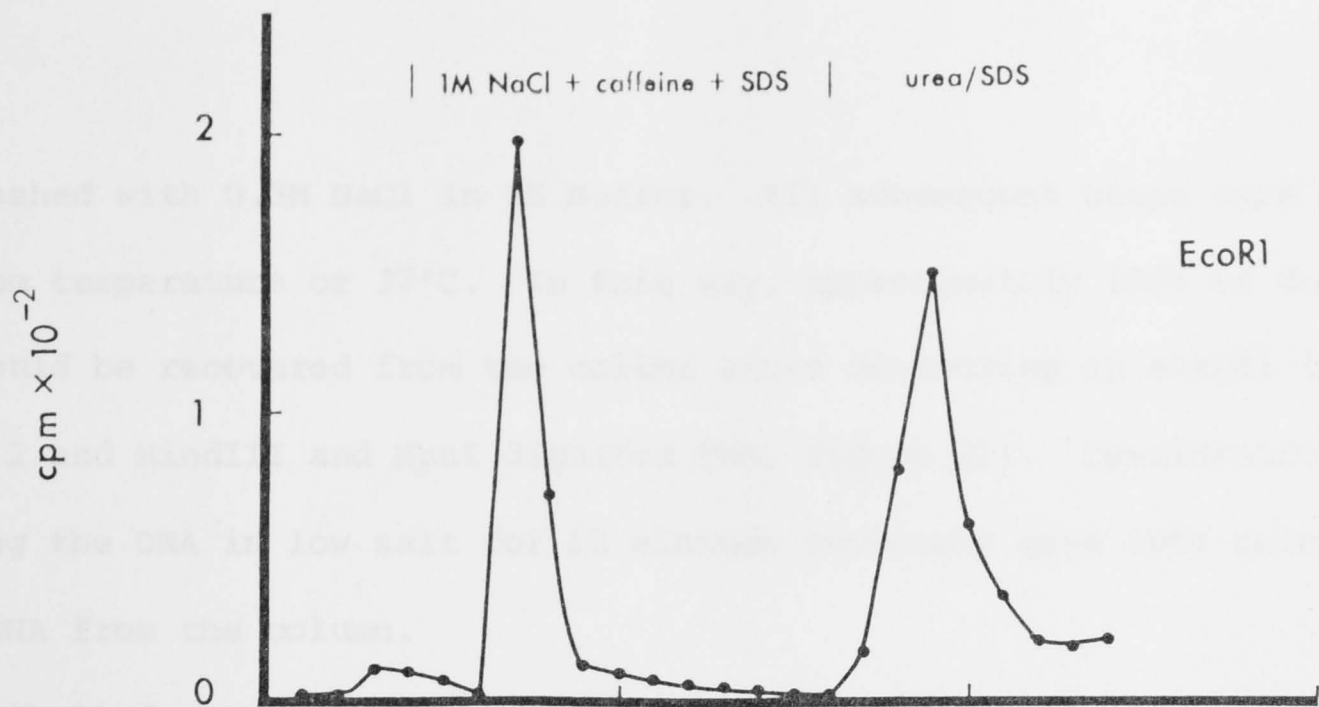


By analogy with virion DNA-protein complex, the protein found on the termini of intracellular completed and replicating viral DNA is most likely to be attached to the 5' end of the DNA strands (see discussion). Results presented so far indicate that the protein is associated with the terminal restriction fragments of replicating DNA. The protein on a replicating DNA molecule could be (i) associated only with parental DNA strands and not newly replicated DNA strands or (ii) associated with all 5' ends of replicating DNA, including the 5' ends of both the newly replicated DNA strand and the parental DNA strand. The following experiments examined whether protein was attached to pulse-labeled single-strands from denatured terminal restriction enzyme fragments. If protein was only on parental DNA strands, (i) above, then no pulse-labeled DNA should bind to BND-cellulose after denaturation of the terminal restriction fragments. However, if protein was attached to newly replicated DNA strands as well as parental DNA strands, DNA from pulse-labeled, denatured restriction fragments should bind to the columns.

Initial experiments were complicated by the fact that only 63% of DNA was recovered from the column, which was consistent with the finding that a maximum of 66% of radioactivity in alkali denatured virion DNA could be recovered from BND-cellulose with 1M NaCl, 2% caffeine and 0.1% SDS in TE buffer (examples, Table 2 and EcoRI-digested DNA, Figure 21). Subsequently, it was found that 100% recovery of alkali denatured DNA could be obtained by the following procedure. The restriction enzyme digested complex in approximately 200  $\mu$ l was made at least 10mM with EDTA and denatured with 0.15M NaOH at room temperature for 5 to 10 minutes and then put on ice. The DNA solution was then diluted at 0°C to 2 ml with TE buffer containing 0.3M NaCl and sufficient acetic acid to bring the solution to approximately pH 8. The DNA was loaded onto a column at 4°C

Figure 21

BND-cellulose chromatography of restriction enzyme-digested, denatured replicating DNA-protein complex. Ad5 DNA was labeled with  $^3\text{H}$ -thymidine for 10 minutes at 20 hr p.i., with either restriction endonucleases EcoRI, HindIII or HpaI, denatured in 0.15M NaOH for 10 min at room temperature, then neutralized by addition of 10 volumes of 0.3M NaCl and TE buffer and the required amount of acetic acid at 0°C. The DNA was then applied to BND-cellulose columns at 4°C and eluted with the indicated buffers at room temperature or 37°C. The concentrations of NaCl, SDS, caffeine, urea and protease were the same as for Figure 15. Top, EcoRI-digested (16,000 cpm); Middle, HindIII-digested (110,000 cpm) and Bottom, HpaI-digested (5,800 cpm), Ad5 DNA-protein complex. A sample from each 1 ml fraction was counted in Triton X scintillation fluid.





and washed with 0.3M NaCl in TE buffer. All subsequent steps were done at room temperature or 37°C. In this way, approximately 100% of denatured DNA could be recovered from the column after denaturing in alkali (e.g. Table 2 and HindIII and HpaI digested DNA, Figure 21). Denaturation by boiling the DNA in low salt for 10 minutes routinely gave 100% recovery of this DNA from the column.

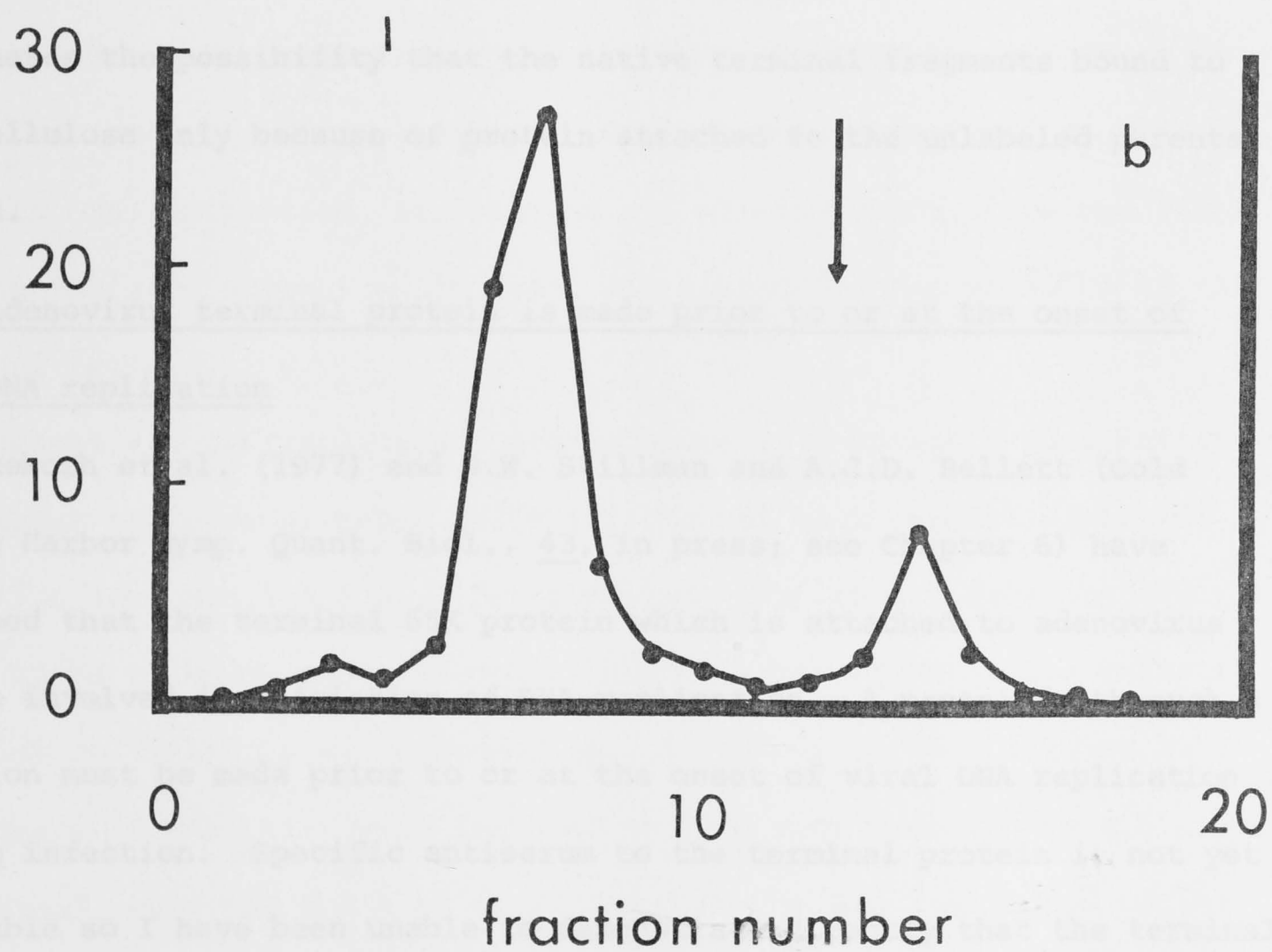
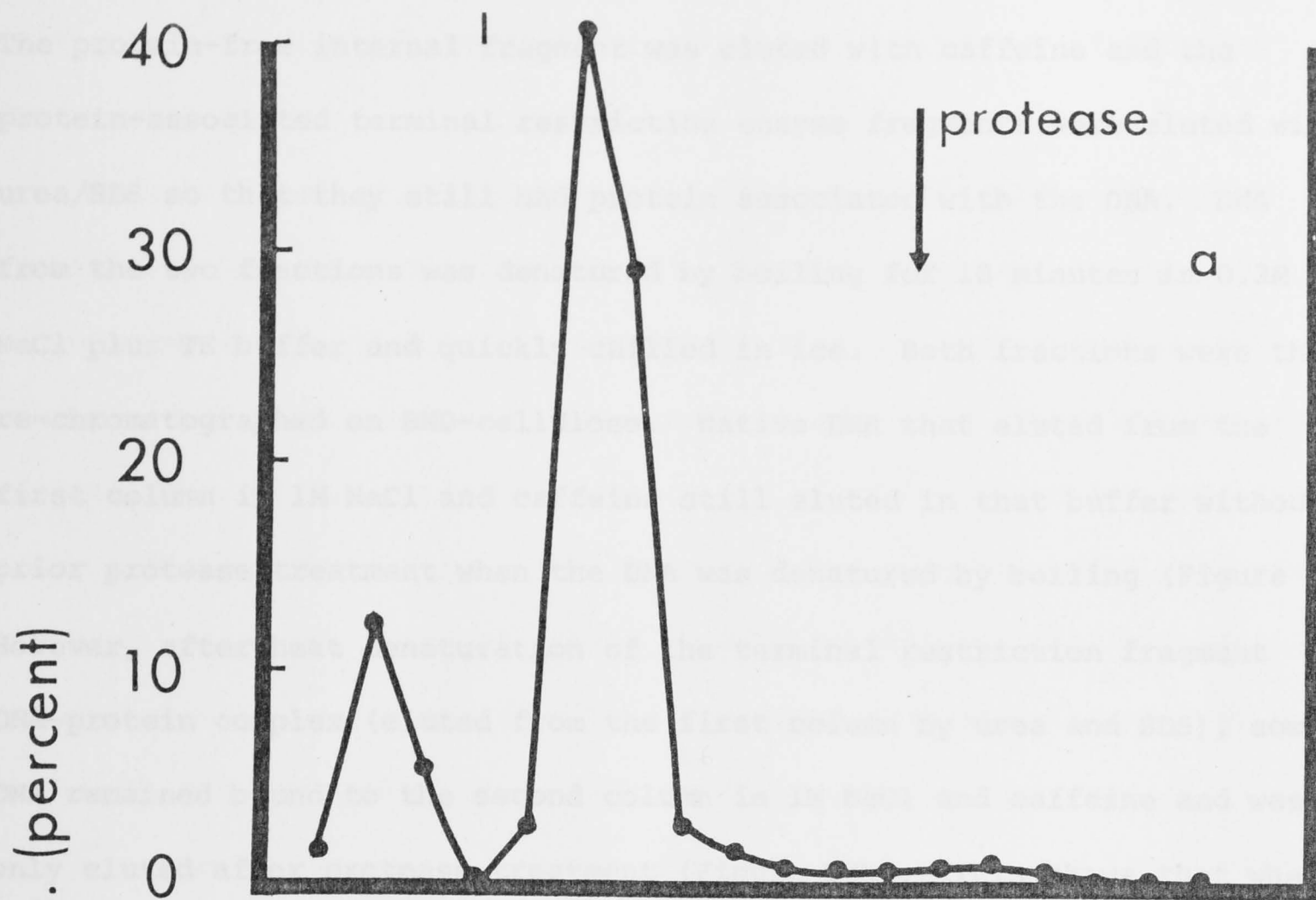
Alkali-denatured, EcoRI-digested, pulse-labeled DNA eluted with a different profile from that of native EcoRI fragments (Figure 21). Approximately 50% of the DNA recovered from the column was eluted with 1M NaCl and caffeine, indicating that half the single-stranded DNA from the terminal restriction fragments is not associated with protein. The other 50% eluted from the column with urea/SDS. Similarly, only 95% of HindIII-digested, denatured pulse-labeled DNA eluted with 1M NaCl and caffeine and 5% remained bound to the column and only eluted with urea/SDS (Figure 21). The terminal HindIII restriction fragments of Ad5 DNA, HindIII G and HindIII I, are 7% and 3% of the whole DNA respectively (Sussenbach and Kuijk, 1977). Since 5%, or half of the radioactivity expected from the native terminal HindIII fragments, eluted from BND-cellulose in urea/SDS, a substantial amount of the growing progeny strands from the terminal restriction enzyme fragments of replicating DNA have protein covalently attached. The retention of denatured, terminal restriction fragments in the presence of 1M NaCl and caffeine is due to the presence of protein, since some HpaI-digested, denatured, pulse-labeled Ad5 DNA could be eluted from BND-cellulose only after protease digestion on the column (Figure 21).

Pulse-labeled, viral DNA that binds to BND-cellulose after denaturation is derived from the denatured terminal restriction fragments, but not from internal denatured fragments (Figure 22). Pulse-labeled DNA-protein complex was digested with endonuclease EcoRI and protein-free and

Figure 22

BND-cellulose chromatography of denatured, EcoRI-digested replicating Ad5 DNA. Replicating Ad5 DNA labeled with  $^3\text{H}$ -thymidine in a 10 min pulse (16,300 cpm) was digested with EcoRI and chromatographed on BND-cellulose as in Figure 20c. DNA not associated with protein (1M NaCl and 2% caffeine eluate: a) and DNA associated with protein (urea/SDS eluate: b) was alcohol-precipitated, denatured by boiling for 10 min in 0.3M NaCl and TE buffer, chilled in ice and then re-chromatographed on BND-cellulose in TE buffer containing 1M NaCl and 2% caffeine. Where indicated, the column was incubated in buffer containing 1 mg/ml protease and the remaining DNA eluted with 1M NaCl and caffeine. Results were expressed as the radioactivity recovered in each 1 ml fraction as a percentage of total radioactivity added to the column.

1M NaCl + caffeine





protein-associated DNA were isolated on BND-cellulose as in Figure 20c. The protein-free internal fragment was eluted with caffeine and the protein-associated terminal restriction enzyme fragments were eluted with urea/SDS so that they still had protein associated with the DNA. DNA from the two fractions was denatured by boiling for 10 minutes in 0.3M NaCl plus TE buffer and quickly chilled in ice. Both fractions were then re-chromatographed on BND-cellulose. Native DNA that eluted from the first column in 1M NaCl and caffeine still eluted in that buffer without prior protease treatment when the DNA was denatured by boiling (Figure 22a). However, after heat denaturation of the terminal restriction fragment DNA-protein complex (eluted from the first column by urea and SDS), some DNA remained bound to the second column in 1M NaCl and caffeine and was only eluted after protease treatment (Figure 22b). This shows that when terminal fragments are isolated from replicating DNA after a short pulse, some of the labeled progeny strands have protein attached. This also eliminates the possibility that the native terminal fragments bound to BND-cellulose only because of protein attached to the unlabeled parental strand.

(c) Adenovirus terminal protein is made prior to or at the onset of DNA replication

Rekosh et al. (1977) and B.W. Stillman and A.J.D. Bellett (Cold Spring Harbor Symp. Quant. Biol., 43, in press; see Chapter 6) have proposed that the terminal 55K protein which is attached to adenovirus DNA is involved in initiation of DNA replication. A protein with such a function must be made prior to or at the onset of viral DNA replication during infection. Specific antiserum to the terminal protein is not yet available so I have been unable to demonstrate directly that the terminal protein is made early by immunofluorescent staining of infected cells.

However, I have obtained evidence which indicates that the protein found on the termini of replicating DNA is produced prior to or at the onset of DNA replication.

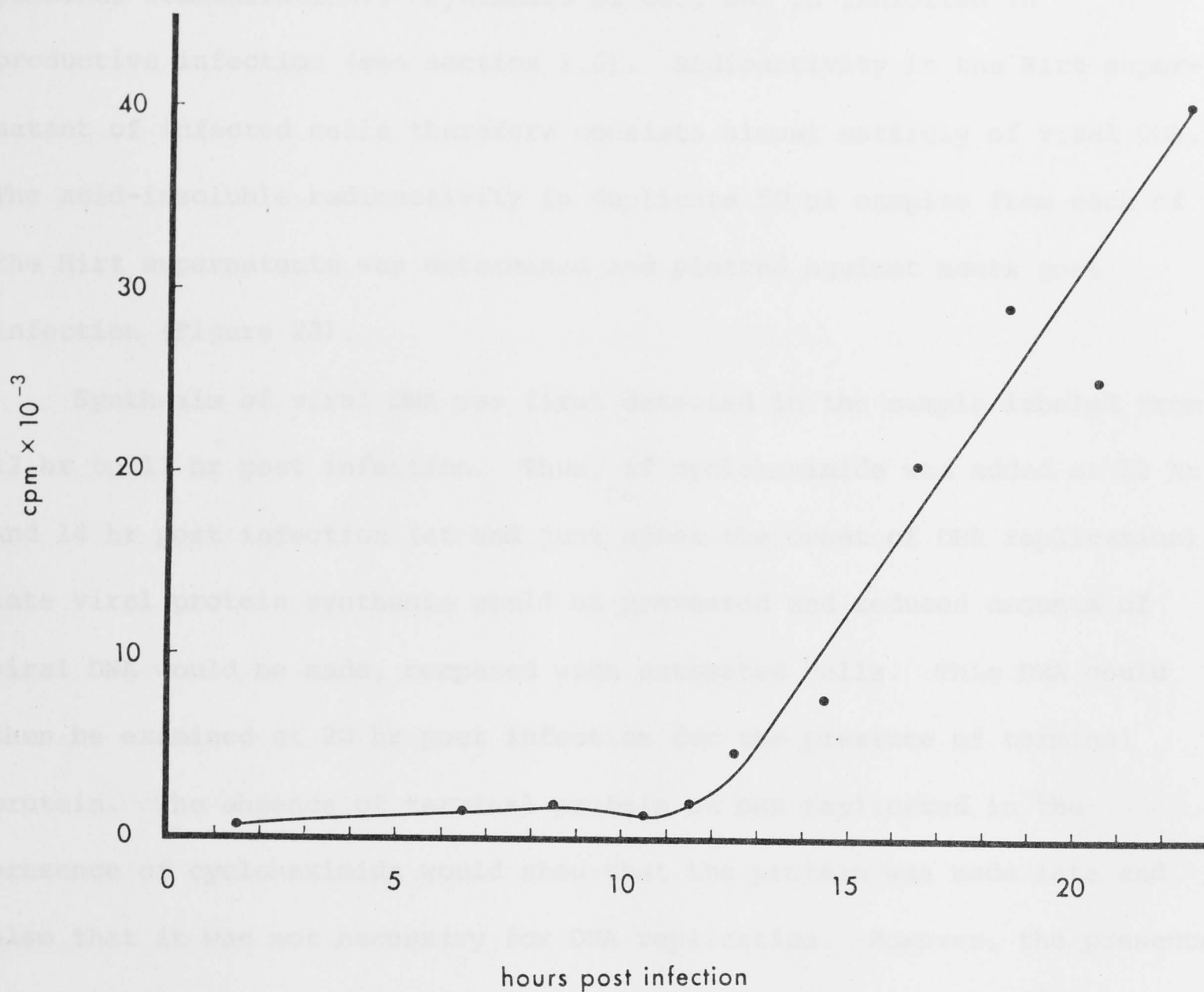
Horwitz et al. (1973) demonstrated that once started, adenovirus DNA replication could continue in the presence of cycloheximide. If cycloheximide was added just after DNA replication had commenced, the amount of viral DNA replication compared to untreated control cells was reduced, but the DNA replication which did occur continued for some time after addition of the drug. The later cycloheximide was added, the more DNA replication was observed, until control levels were obtained even in the presence of cycloheximide. This indicated that proteins necessary for viral DNA replication were made prior to DNA replication, and continued to be synthesized during the first hour or two of DNA replication, but were then in sufficient amount to support continued DNA replication in the absence of further protein synthesis. I have used this phenomenon to demonstrate that the terminal protein is made early or at the onset of DNA replication. Cycloheximide was added at the onset of DNA replication, replication was allowed to continue and viral DNA was isolated several hours later and tested for the presence of the terminal protein.

Adenovirus DNA replication starts about 7 to 10 hours post infection in spinner cultures of human cells infected at high multiplicities of infection (see section 1.6). But viral DNA replication does not begin until 11 to 12 hours post infection in monolayers of HEK cells infected at a low multiplicity of infection (m.o.i.) of 10 iu/cell (Figure 23). Monolayers of HEK cells were infected at time zero with Ad5 at m.o.i. of 10 iu/cell, absorbed for 1 hr at 37°C and medium was then added to the infected cells. At various times post infection, the cells were pulse-labeled with <sup>3</sup>H-thymidine for 1 hr at 37°C and then viral DNA was isolated

Figure 23

Time course of Ad5 DNA replication after infection of monolayers of human HEK cells. Cells were infected by Ad5 at a multiplicity of infection of 10 iu/cell, absorbed at 37°C for 1 hr and then at various times post infection, pulse-labeled with  $^3\text{H}$ -thymidine (20  $\mu\text{Ci/ml}$ ) for 1 hr at 37°C. Viral DNA was prepared by the modified procedure of Hirt (1967), described in section 3.2.  $^3\text{H}$ -acid-insoluble radioactivity was determined in duplicate 50  $\mu\text{l}$  samples of the Hirt supernatant. The results are shown plotted at the mid-point of each labeling period.





by the procedure described by Hirt (1967) (see Chapter 3.2). Cell DNA is preferentially precipitated by the Hirt method (Hirt, 1967), while more than 80% of adenovirus DNA remains in the supernatant (A.J.D. Bellett, personal communication). Synthesis of cell DNA is inhibited in productive infection (see section 1.6). Radioactivity in the Hirt supernatant of infected cells therefore consists almost entirely of viral DNA. The acid-insoluble radioactivity in duplicate 50  $\mu$ l samples from each of the Hirt supernatants was determined and plotted against hours post infection (Figure 23).

Synthesis of viral DNA was first detected in the sample labeled from 12 hr to 13 hr post infection. Thus, if cycloheximide was added at 12 hr and 14 hr post infection (at and just after the onset of DNA replication), late viral protein synthesis would be prevented and reduced amounts of viral DNA would be made, compared with untreated cells. This DNA could then be examined at 20 hr post infection for the presence of terminal protein. The absence of terminal protein on DNA replicated in the presence of cycloheximide would show that the protein was made late and also that it was not necessary for DNA replication. However, the presence of terminal protein on DNA replicated in the presence of cycloheximide would show that the protein was made prior to 12 hr or 14 hr post infection and is consistent with a proposed role for the protein in viral DNA replication (see Chapter 6).

HEK cells were infected with Ad5. At either 12 hr or 14 hr post infection, 30  $\mu$ g/ml of cycloheximide was added (Horwitz et al., 1973) and the cells were incubated at 37°C for a further 4 hr or 6 hr. The cells were then pulse-labeled with  $^3$ H-thymidine for 10 minutes and viral DNA isolated as described in section 5.2a. When cycloheximide was added at 12 hr post infection and pulse-labeled at 16 hr post infection, incorporation of  $^3$ H-thymidine was 10% of that in infected cells without

cycloheximide; but when cycloheximide was added at 14 hr post infection and cells pulse-labeled at 20 hr post infection, viral DNA replication was 30% of that in infected cells without drug. Viral DNA isolated at 16 hr post infection, after addition of cycloheximide at 12 hr (the onset of viral DNA replication), behaved as DNA-protein complex when run on a BND-cellulose column (Figure 24a). Both molecules completed during the pulse (eluted by 1M NaCl + SDS) and molecules still replicating at the end of the pulse (eluted by 1M NaCl + caffeine) had protein attached after 4 hr of DNA replication in the presence of cycloheximide. The viral DNA isolated at 20 hr post infection, after addition of cycloheximide at 14 hr post infection, was cut with restriction endonuclease EcoRI and run on BND-cellulose (Figure 24b). Table 4 shows that only the internal fragment EcoRI C was eluted with 1M NaCl and caffeine and the terminal fragments EcoRI A and B eluted with urea and SDS, indicating that protein was on the ends of all replicating and completed DNA molecules labeled in a 10 minute pulse after 6 hr of replication in the presence of cycloheximide. Late protein synthesis was not detected in cycloheximide-treated cells, in agreement with Horwitz et al. (1973). Figure 25 shows that by indirect fluorescent antibody staining, reduced amounts of early viral antigens were detected at 20 hr in cells that had been treated with cycloheximide from either 12 or 14 hr post infection, but late viral antigens were not detected at all. Furthermore, when cells were labeled with  $^{14}\text{C}$ -leucine at 16 hr post infection, after addition of cycloheximide at 14 hr post infection, protein synthesis was inhibited by 92% compared with that in infected cells not treated with cycloheximide. Thus these experiments indicate that the protein found on the termini of replicating viral DNA in cycloheximide-treated adenovirus-infected cells was synthesized before the drug was added, and therefore prior to or at the onset of viral DNA replication.



Figure 24

Presence of terminal protein on Ad5 DNA replicating in the presence of cycloheximide. BND-cellulose chromatography of (a) replicating DNA from Ad5-infected cells incubated in cycloheximide from 12 hr to 16 hr post infection and then pulse-labeled with  $^3\text{H}$ -thymidine for 10 min (1,000 cpm); (b) EcoRI-digested replicating Ad5 DNA from cells incubated in cycloheximide from 14 hr to 20 hr post infection and then pulse-labeled for 10 min (3,000 cpm).

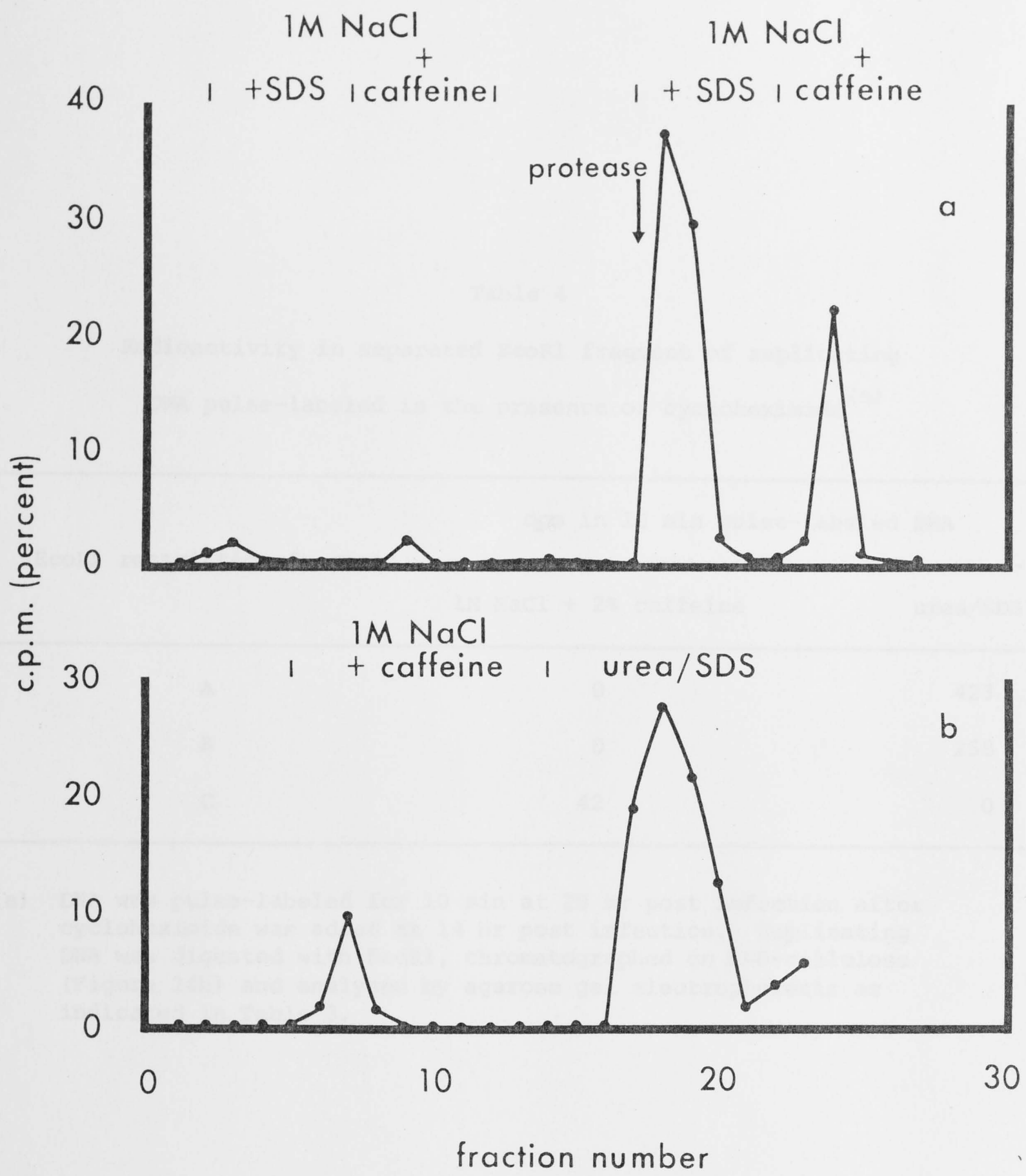


Table 4

Radioactivity in separated EcoRI fragment of replicating  
DNA pulse-labeled in the presence of cycloheximide<sup>(a)</sup>

EcoRI restriction fragment	cpm in 10 min pulse-labeled DNA	
	1M NaCl + 2% caffeine	urea/SDS
A	0	423
B	0	258
C	42	0

- (a) DNA was pulse-labeled for 10 min at 20 hr post infection after cycloheximide was added at 14 hr post infection. Replicating DNA was digested with EcoRI, chromatographed on BND-cellulose (Figure 24b) and analysed by agarose gel electrophoresis as indicated in Table 3.

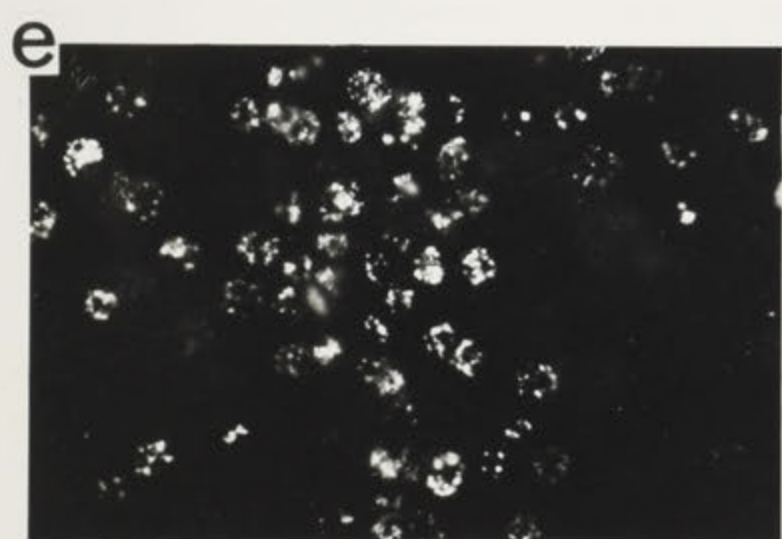
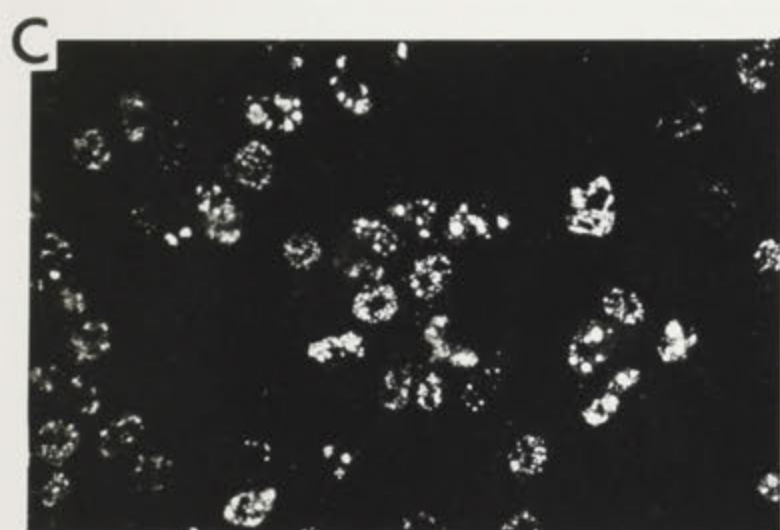
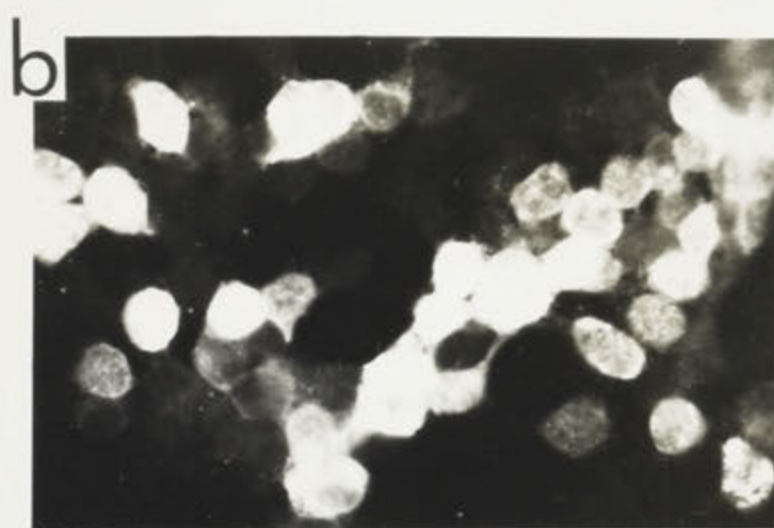
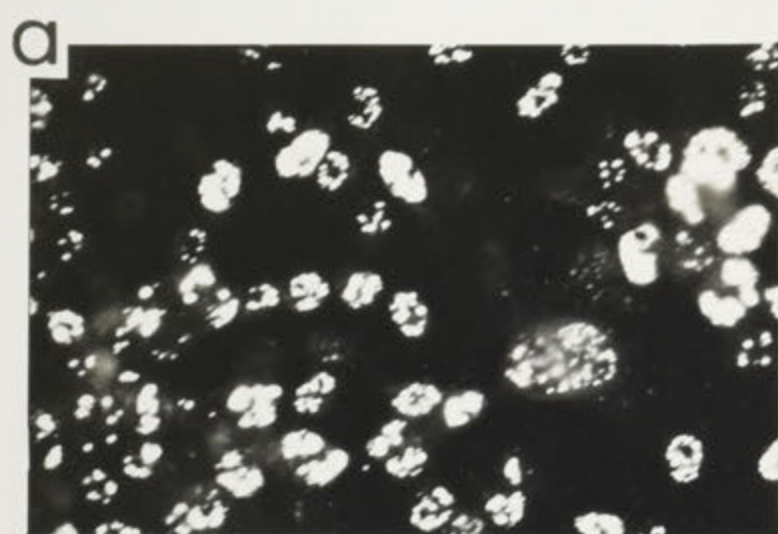


Figure 25

Effect of cycloheximide on protein synthesis in Ad5-infected cells by fluorescent antibody staining (Philipson, 1961). Cells were stained with either early antigen-specific 'P' antiserum which reacts with DNA-binding protein and T antigen, or late antigen-specific 'V' antiserum at 20 hr p.i. (a) and (b), Ad5-infected cells without cycloheximide; (c) and (d), cycloheximide (30  $\mu$ g/ml added from 12 hr to 20 hr p.i.; (e) and (f), cycloheximide added from 14 hr to 20 hr p.i.; (g) and (h), uninfected HEK cell controls.

P antiserum

V antiserum



#### 5.4 Discussion

Using the simple assay for adenovirus DNA-protein complex described in Chapter 4, I have demonstrated that a protein is associated, possibly covalently, with labeled strands of terminal restriction enzyme fragments of pulse-labeled, intracellular DNA. Analysis of the bound, labeled DNA-protein complex on a column by treatment with protease and elution with salt, followed by caffeine, shows that protein is on the ends of molecules that were still replicating when the cells were lysed. Protein associated with the pulse-labeled strand at the end of replicating DNA molecules could be attached to either the 3' terminal single strands of molecules that had just terminated during the pulse, or the 5' terminal single strands of molecules that had just initiated during the pulse. The binding of pulse-labeled, single strands to BND-cellulose indicates that the protein is associated with newly replicated DNA and not just with parental DNA strands. Available evidence indicates that the protein is associated with the 5' ends of newly initiated viral DNA. Intracellular viral DNA is sensitive to digestion with the 3' specific exonuclease III (Chapter 3; Estes, 1978), but is not degraded by the 5' specific  $\lambda$  exonuclease (Estes, 1978), which indicates that intracellular viral DNA has a blocking group attached to the 5' end. This has also been demonstrated for virion DNA, even after protease digestion of the DNA-protein complex (Carusi, 1977). The most simple conclusion is that the terminal protein is associated with the 5' end of intracellular DNA, especially since it has been demonstrated that the 55K terminal protein is associated with the 5' ends of virion DNA (Carusi, 1977; Rekosh et al., 1977).

The pulse-labeled strands from terminal restriction fragments that bound to BND-cellulose, did not form a sharp enough band when run on agarose gels (Hayward, 1972) to determine directly whether they were from the 5' end of the DNA strands (data not shown). This is probably due to



the amount of handling of replicating DNA prior to running on a strand separation gel. I have found it important, even with virion DNA, not to introduce single-strand breaks into the DNA in order to get sharp bands of denatured restriction fragments of DNA on agarose gels.

Robinson and Bellett (1974) obtained preliminary evidence for the association of protein with replicating DNA based on sedimentation studies in sucrose gradients. Girard et al. (1977) have demonstrated that terminal fragments of intracellular DNA, pulse-labeled for times less than the time of replication of the viral DNA molecule, do not enter agarose gels due to the association of protein with them. Replicating DNA also appeared to have protein associated with each terminus, causing circularization of DNA molecules viewed in the electron microscope. Using a filter binding assay for adenovirus DNA-protein complex, A.J. Robinson, D.H. Coombs and G.D. Pearson (personal communication) have also demonstrated that pulse-labeled replicating DNA has protein associated with the terminal restriction enzyme fragments.

It is unlikely that the protein found on the termini of replicating DNA is different from the 55K protein found on the termini of virion DNA (Rekosh et al., 1977). Rekosh et al. (1977) have noted the similarity in molecular weight between this protein and the adenovirus tumor (T) antigen, a protein which is produced early in infection and by analogy with other tumor virus T antigens, is also likely to be involved in viral DNA replication (section 1.10). The stability of the DNA-protein linkage in the replicating DNA-protein complex to 8M urea and 1% SDS, 0.1M NaOH, GuHCl and after boiling suggests that the protein is covalently bound to single strands at the ends of replicating DNA molecules. Several attempts during the course of this work to label the protein found on the termini of replicating DNA in vivo with  $^{35}\text{S}$  methionine have not been successful (see discussion, Chapter 4).



I have presented evidence that the protein that is associated with the ends of replicating DNA is synthesized prior to or at the onset of viral DNA replication, by inhibiting further protein synthesis after the onset of viral DNA replication with cycloheximide. A terminal protein is associated with the 5' terminus of virion DNA and evidence discussed above suggests a similar association of a terminal protein with the 5' ends of replicating DNA. The 5' termini of adenovirus DNA have been shown to be the origins of DNA replication (Horwitz, 1976; Ariga and Shimojo, 1977; Lechner and Kelly, 1977; Sussenbach and Kuijk, 1978). The fact that the protein is made at or prior to the onset of viral DNA replication and is likely to be associated with the origin of DNA replication is consistent with a model for DNA replication that is discussed in Chapter 6.

## CHAPTER 6

## DISCUSSION

The overall mechanism of adenovirus DNA replication during productive infection of cells has been determined in the past six years to be the strand displacement mechanism shown in Figure 7 in Chapter 1. Except for the virus-coded DNA-binding protein, the proteins involved in viral DNA replication have not been identified and the function of the inverted terminal repetition in the DNA, which contains the origin of DNA replication, has not been determined. In addition, the overall mechanism of DNA replication does not explain how the ends of the linear DNA molecule are replicated, as first pointed out by Bellett and Younghusband (1972).

Three possible mechanisms for initiation of adenovirus DNA replication have been examined. Formation of circular or concatemeric intermediates (Robinson and Bellett, 1974; Girard et al., 1977) would allow synthesis of an RNA molecule at the origin and its subsequent removal and replacement by repair synthesis, like bacteriophage  $\lambda$  DNA replication (Chapter 1). Covalently closed circles or concatemers have not been observed during adenovirus DNA replication and there is no evidence for non-inverted terminal repeats in replicating DNA which would allow the formation of such intermediates (Horwitz, 1971; Bellett and Younghusband, 1972; Sussenbach et al., 1972; van der Eb, 1973; Levine et al., 1976; Chapter 3 of this work). Replicating DNA isolated in the absence of protease digestion can appear circular when viewed in the electron microscope (Girard et al., 1977) due to protein-protein interactions. These authors have proposed that the protein-mediated circular molecules are involved in the initiation of viral DNA replication, but unless the DNA is covalently closed at some stage, circularization of the complex is not relevant to DNA replication. It is possible that adenovirus DNA could exist inside the virus and the cell as a covalently closed circular molecule complexed non-covalently with terminal proteins and that all methods of isolation induced the proteins to nick the DNA, leaving the

proteins covalently attached to one strand on each end (Robinson et al., 1973). This could be a similar situation to the relationship between bacterial plasmids and their relaxation proteins (section 1.1), although only one protein is covalently attached to plasmid DNA after nicking of the circular DNA and methods are available to isolate the plasmid DNA as a covalently closed circle. In spite of many attempts using different isolation procedures, it has not been possible to demonstrate that replicating adenovirus DNA ever exists as a covalently closed circular structure. Although the circularization model cannot be excluded, there is no evidence in favour of it and it is no longer considered a serious possibility.

Secondly, a mechanism of initiation of DNA replication has been proposed in which the parental 3' terminus forms a hairpin loop, allowing the 3'-hydroxyl to prime DNA replication, with a subsequent strand switch and inversion of the terminus by endonuclease nicking and repair synthesis of the parental 3' terminal sequence (Figure 9, Chapter 3). Evidence has been presented in Chapter 3 which is inconsistent with this mechanism for adenovirus DNA replication. The non-palindromic terminal repetition does not invert during replication, since none of the intracellular DNA molecules form circles after digestion with exonuclease III and annealing (section 3.3a). In vivo double labelling experiments have failed to detect the repair synthesis at the 3' end of the parental strand required by the model (section 3.3c) and there was no evidence for the existence in replicating DNA of self-complementary sequences of joined parental and progeny DNA predicted by the model (section 3.3b). In addition, the DNA sequence of the inverted terminal repetition of adenovirus types 2 and 5 (Steenbergh et al., 1978; J.R. Arrand and R.J. Roberts, personal communication) is incompatible with formation of self-



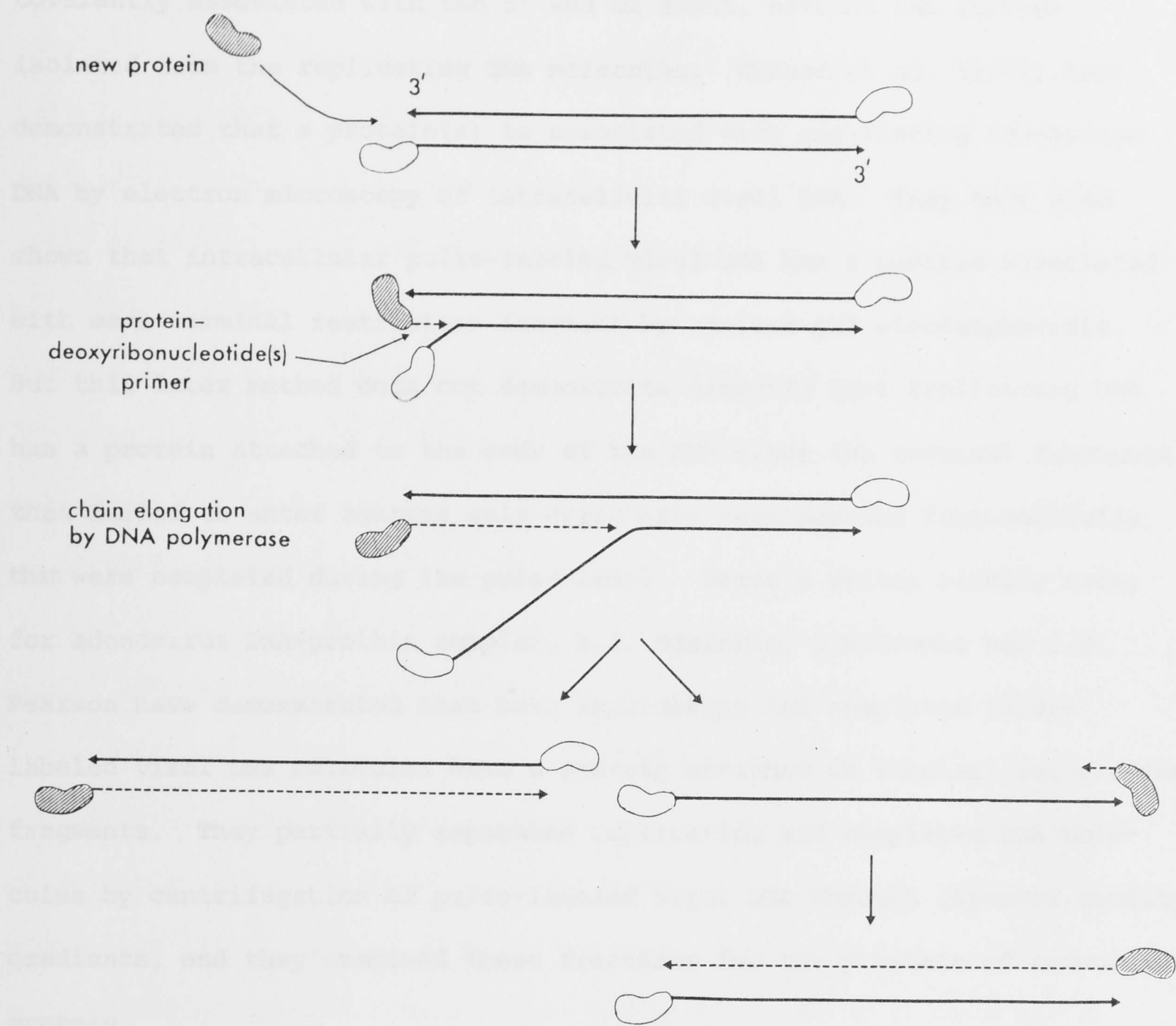
complementary hairpin loops. Recently, Sussenbach and Kuijk (1978b) have also presented evidence inconsistent with this model. Their data show that a strand switch within the terminal restriction fragments of newly replicated viral DNA does not occur.

A third model for the initiation of DNA replication in adenoviruses was proposed by Rekosh et al. (1977) and is consistent with all known data concerning adenovirus DNA replication. The model suggests that a newly synthesized protein, similar to the 55K protein found on the ends of virion DNA, covalently binds the 5' terminal nucleotide and the protein-nucleotide complex acts as a primer for DNA polymerase. This model has been modified (Figure 26) to include the possibility that the protein recognizes the origin of replication and synthesizes a short primer sequence, which remains covalently bound to the protein and becomes similarly attached to replicating DNA as the primer is elongated by DNA polymerase. Although this mechanism is less likely, primases of this type (which do not become covalently bound to the DNA) have been found in a number of prokaryote DNA replication systems (Wickner, 1977; Kornberg, 1977; Scherzinger et al., 1977; Rowen and Kornberg, 1978a,b) and have also been suggested for initiation of short DNA chains during polyoma DNA synthesis in isolated nuclei (Eliasson and Reichard, 1978). The proposed adenovirus-priming protein would utilize only deoxyribonucleotides for priming.

Evidence has been presented which demonstrates that a protein is associated, probably covalently, with the labeled strands from the terminal restriction fragments of replicating adenovirus DNA (Chapter 5), by the use of an assay system for both mature and replicating DNA-protein complex on benzoylated, naphthoylated DEAE cellulose, described in Chapter 4. As discussed in Chapter 5, it is likely that this protein is covalently attached to the 5' ends of replicating DNA, which are the origins of viral

Figure 26

A model for the mechanism of initiation of adenovirus DNA replication. This model was proposed by Rekosh et al. (1977) as a possible role for the terminal protein in priming DNA replication of the progeny strand, and has been modified to include the possibility that the terminal protein acts as a 'primase', similar to prokaryote primases (see text), or simply covalently binds the terminal nucleotide which then acts as the primer as originally proposed. The single-strand intermediate resulting from displacement of one parental strand could form a circle with a double-stranded 'pan-handle' by annealing of the complementary sequences at its ends, as proposed by Lechner and Kelly (1977) (Figure 7) and hence a double-strand recognition site at the origin of replication would be maintained. This version of the model suggests a function for the inverted repetition as well as the terminal protein. Initiation could occur at either end of the parental duplex molecule.





DNA replication (Horwitz, 1976; Ariga and Shimojo, 1977; Sussenbach and Kuijk, 1978). However, it is still necessary to show that the protein is covalently associated with the 5' end of short, nascent DNA strands isolated from the replicating DNA molecules. Girard et al. (1977) have demonstrated that a protein(s) is associated with replicating adenovirus DNA by electron microscopy of intracellular viral DNA. They have also shown that intracellular pulse-labeled viral DNA has a protein associated with each terminal restriction fragment by agarose gel electrophoresis. But this later method does not demonstrate directly that replicating DNA has a protein attached to the ends of the DNA since the terminal fragments that failed to enter agarose gels could have been derived from molecules that were completed during the pulse-label. Using a filter binding assay for adenovirus DNA-protein complex, A.J. Robinson, D.H. Coombs and G.D. Pearson have demonstrated that both replicating and completed pulse-labeled viral DNA molecules have a protein attached to terminal restriction fragments. They partially separated replicating and completed DNA molecules by centrifugation of pulse-labeled viral DNA through glycerol density gradients, and they examined these fractions for the presence of associated protein.

Proteins covalently bound to the ends of bacteriophage or other viral genomes have been implicated as being involved in replication. A protein is firmly (possibly covalently) attached to the ends of both virion and replicating bacteriophage  $\phi$ 29 DNA (Ortin et al., 1971; Harding and Ito, 1976; Salas et al., 1978) and Harding and Ito (1976) have demonstrated that the protein is synthesized prior to or during phage DNA replication. The terminal protein is probably the same as the phage gene 3 protein (Salas et al., 1978; Harding et al., 1978). Mutants of the phage gene 3 have a DNA-protein complex which is temperature-sensitive for transfection

(Yanofsky et al., 1976) and mutants in this gene are also defective in phage DNA replication, possibly at the initiation step (McGuire et al., 1977).

A protein, designated VPg, has been found on the 5' termini of poliovirus RNA (Flanegan et al., 1977; Lee et al., 1977; Nomoto et al., 1977). The protein is linked to short nascent strands of RNA from replicative intermediates (Pettersson et al., 1978) and has been implicated in initiation of RNA replication (Flanegan et al., 1977; Nomoto et al., 1977; Pettersson et al., 1978), possibly by a protein-nucleotide complex acting as a primer for a primer-dependent RNA polymerase (Flanegan et al., 1977). Thus, covalent linkage of proteins to nucleotide sequences could be involved in the initiation of replication of a number of genomes.

It is unlikely that the protein on the ends of replicating adenovirus DNA is different from the 55K protein on the 5' ends of the strands of virion DNA (Rekosh et al., 1977), although their identity has not yet been demonstrated. That the virion protein is coded by the virus is suggested by molecular weight differences found between the terminal proteins from adenovirus types 5 and 12 grown in the same cell type (D.M.K. Rekosh and W.C. Russell, personal communication). As mentioned in Chapter 5, one requirement for a protein to be involved in viral DNA replication is that it is produced early in adenovirus infection, prior to the onset of viral DNA replication. I have demonstrated in Chapter 5 that the terminal protein found on replicating adenovirus DNA is produced prior to or at the onset of viral DNA replication. Direct demonstration that the protein is made early in infection is made difficult by the fact that it has only been purified and identified by virtue of its attachment to viral DNA, and there is no assay for free intracellular protein. Consequently, the exact time course of synthesis of the terminal protein has not been

determined. The use of specific antisera to the terminal protein and localization of the map position for the terminal protein coding region will be necessary to determine the time course of synthesis of the terminal protein, by fluorescent antibody staining or detection of mRNA levels in infected cells respectively.

Sequences complementary to early mRNA in both adenovirus types 2 and 5 DNA comprise four discrete regions of the genome (Sharp and Flint, 1976; Chow et al., 1977; Flint, 1977; Philipson, 1977; Berk and Sharp, 1978) and the 'spliced' cytoplasmic RNAs coded by these regions have recently been mapped (Berk and Sharp, 1978). There are, in general, more cytoplasmic RNAs coded by the early regions of adenovirus type 2 than are necessary to code for the identified early polypeptides (Berk and Sharp, 1978) and it is not possible to assign a region where the terminal protein is likely to be coded. Rekosh et al. (1977) have noted that the 55K terminal protein has a similar molecular weight to the adenovirus tumor antigen, which is coded by early mRNA from the left hand end of the r strand of the adenovirus genome (see Figure 8). This is the only early protein so far identified in both productively infected and transformed cells, which has a molecular weight similar to the 55K terminal protein (Lewis et al., 1976; Gilead et al., 1976b; Levinson and Levine, 1977a; Flint 1977; Harter and Lewis, 1978). Comparison of these two proteins by peptide analysis could indicate whether they are related in any way.

An mRNA species coding for the adenovirus structural protein IX is transcribed from the 'early' region at the left hand end of the r strand (Chow et al., 1977; Pettersson and Mathews, 1977). Although, unlike the other (late) virion structural proteins, protein IX and its mRNA are produced 'early' in infection, most synthesis of protein IX occurs after the onset of DNA replication (Persson et al., 1978). The addition of cycloheximide early in infection, which blocked transcription of the



'authentic' late protein genes, did not prevent synthesis of mRNA for protein IX. This has led to the possible identification of a new class of viral transcription products in which the genes are transcribed after the 'authentic early' genes, but before the 'authentic late' genes (Persson et al., 1978). The adenovirus terminal protein could well fit into either the 'early' class or the protein IX class of gene products, as experiments described in Chapter 5 have shown that the terminal protein is made before the synthesis of the 'authentic late' structural proteins.

The data presented here are consistent with, although do not prove, the model for adenovirus DNA replication proposed by Rekosh et al. (1977) and shown in Figure 26. The protein found on the termini of replicating viral DNA molecules is synthesized early in infection or at the onset of DNA replication. It has been proposed that this protein is involved in the initiation of viral DNA replication. Some early proteins of DNA tumor viruses that are involved in viral DNA replication are also involved in stimulation of host cell DNA replication and cellular transformation (section 1.10), so it will be interesting to determine the role, if any, of the terminal protein in adenovirus DNA replication. As indicated in Chapter 1, an understanding of the mechanism by which adenovirus early proteins are able to initiate viral DNA replication could provide insight into the mechanism of adenovirus-induced cell transformation of non-permissive cells.

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